Exploration of OX-2 function in tolerance induction and graft acceptance using an anti-mouse OX-2 monoclonal antibody

by

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A thesis submitted in conformity with the requirements for degree of Master of Science.
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Portal venous (pv) peri-transplant immunization with donor bone marrow cells, or dendritic cells derived from bone marrow cultures, along with cyclosporine A treatment (10mg/Kg), leads to antigen-specific increased renal allograft survival in comparison to graft recipients receiving iv donor-specific transfusion. This increased survival is associated with characteristic changes in cytokine production from recipient T cells restimulated in vitro with donor antigen. A suppressive subtractive hybridization approach has shown that there are a number of other genes which are differentially expressed as a result of pv immunization, and which might in turn play a role in regulation of transplant rejection. One of these shows sequence homology to a previously described gene encoding a molecule expressed on rat dendritic cells (MRC OX-2), which in turn has significant sequence homology with genes encoding the co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2). Our laboratory has hypothesized that OX-2 is functionally important in tolerance induction/immunoregulation, and plays a key role in the increased graft survival seen in our transplant model. The tolerizing (negative) signal delivered to the T-cells can be monitored, in at least some instances, by the production of type 2 cytokines. In support of this idea, it has been shown that anti-rat OX-2 reversed graft prolongation in mice following pv immunization.

One criticism raised to the preliminary data we reported was that we had relied on an unexpected cross-reactivity between murine and rat OX-2 to define an immunosuppressive role for OX-2 expression. One specific aim of the current project was to raise and characterize monoclonal antibodies (mAbs) specific to murine OX-2. A second goal of the current work was to characterize the functional importance of expression of OX-2 on graft survival using these anti-mouse OX-2 mAbs. Murine OX-2 mAbs raised in outbred rats were characterized as follows. Western blots were performed using both mouse brain and thymus extracts with the relevant mAbs and the appropriate control antibodies. These results revealed a difference in the size of the band detected in the two tissues, with mouse brain extracts having a band at molecular weight ≈ 33 Kda, while the band in mouse thymus extracts migrated at ≈ 48Kda, as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. These results are in good agreement with the previously published data of Barclay et al for rat OX-2. A difference in the apparent size of this molecule in different tissues is probably a function of differential glycosylation.

FACS analysis was performed using anti-OX-2 mAbs and fresh peripheral blood leukocytes (PBL) or fresh spleen dendritic cells isolated after adherence and overnight incubation. Both cell
preparations stained with anti-OX-2 mAbs at numerical values consistent with independent analyses of their dendritic cell content. A final, more definitive, strategy to confirm that these mAbs did indeed detect murine OX-2 used a vector construct I prepared in which full length OX-2 was inserted into a stable (pBK) expression vector, and CHO cells were transfected with this vector. Stably transfected CHO cells stained with anti-OX-2 mAbs, with no detected expression in cells transfected with control vector as it has been shown by the immunohistochemical staining (3,3-Diaminobenzidine).

Previous data from our laboratory have shown that PV infusion of DCs is a potent means of inducing tolerance in experimental animals. In a final series of experiments I asked whether the newly produced mAbs to murine OX-2 could modify the immune response of allografted responder cells either in vivo in a graft rejection model, or in vitro. The response in vitro was monitored by measuring cytokine production in spleen cells stimulated in an allogeneic mixed leukocyte reaction (MLR). The mouse responder spleen cells (C3H) that were used were derived from mice treated 4 days earlier by PV infusion of C57BL/6 cells, and thus showed a preferential polarization to type-2 cytokine production rather than type-1 cytokine production. While there was no change in cytokine production seen when these cells were incubated with isotype control IgGs, incubation in the presence of the anti-OX-2 mAbs reversed the polarization in cytokine production, with type-1 cytokine now becoming predominant. In addition, anti-OX-2 delivered in vivo to PV immunized mice reversed increased graft survival and the polarization to type-2 cytokine production in cells taken from these animals. Additional data in Chapter 2 showed that continued OX-2 expression was needed for prolongation of graft survival, and/or polarization in cytokine production. This may represent a role for OX-2 signaling at multiple stages in development of the allo-immune response (and its regulation) after pv immunization.

In conclusion, the experiments described in this thesis have highlighted an important role played by the OX-2 molecule in tolerance induction following pv immunization. The anti-murine OX-2 mAbs described will be an asset in future studies to explore the mechanism of, and role of OX-2 expression in, tolerance induced in PV immunized mice. Understanding the mechanisms by which unresponsiveness is maintained is essential for future clinical application of the approaches described.
Acknowledgements

The work presented in this thesis is the result of the contributions of many individuals. I’m indebted to my supervisor, colleagues and friends. I would like to extend my appreciation and sincere gratitude to Dr. Reginald Gorczynski, not only for his tutelage but also for his endless enthusiasm and inspiration. It would not be an overstatement to say that none of this would have been possible without him.

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<tr>
<td>Ag</td>
<td>Antigen</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen Presenting Cell</td>
</tr>
<tr>
<td>mAb</td>
<td>Monoclonal antibody</td>
</tr>
<tr>
<td>pv</td>
<td>portal venous</td>
</tr>
<tr>
<td>iv</td>
<td>intravenous</td>
</tr>
<tr>
<td>ILS</td>
<td>Interleukins</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>MRC OX-2</td>
<td>A member of immunoglobulin supergene family</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>Allo(Ags)</td>
<td>Antigens belonging to genetically different individuals of the same species</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-Lymphocyte</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster determinant of antigens used to characterize a cell surface marker</td>
</tr>
<tr>
<td>CD28</td>
<td>Antigen of immunoglobulin supergene superfamily expressed on T-cells-receptor for B7 group of molecules</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Alternative receptor for B7 molecules expressed on activated T cells (CD125)</td>
</tr>
<tr>
<td>B7 family</td>
<td>A costimulatory family composed of B7.1 (CD80) and B7.2 (CD86)</td>
</tr>
<tr>
<td>SIg</td>
<td>Surface immunoglobulin</td>
</tr>
<tr>
<td>CD3</td>
<td>Molecular complex co-expressed with TCR, forming signaling complex</td>
</tr>
<tr>
<td>GrB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>ICEs</td>
<td>Interleukin - 1 B - converting enzyme family proteases</td>
</tr>
<tr>
<td>FasL</td>
<td>Fas Ligand, surface molecule of the tumor necrosis factor (TNF) gene family.</td>
</tr>
<tr>
<td>Fas (CD95)</td>
<td>A cell surface molecule of the TNF receptor family</td>
</tr>
<tr>
<td>NKs</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>Mφ</td>
<td>Macrophages</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
</tr>
<tr>
<td>HLA</td>
<td>Human Leukocyte Antigen.</td>
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DST: Donor Specific Transfusion
MLR: Mixed Lymphocyte Reaction, used to study the proliferation of T-cells
DTH: Delayed Type Hypersensitivity
TGFB: Transforming Growth Factorβ
STATs: Signal-transducing transcriptional activators
CD11c: gp150,95, surface molecule expressed on surface of myeloid cells
LFA-3/CD58: Member of immunoglobulin supergene family. Leukocyte function-associated antigen-3, binds CD2 adhesion molecule
ICAM-1/CD54: Member of immunoglobulin supergene family. intracellular adhesion molecule. binds CD11a/CD18 (LFA-1) and CD11b/CD18 (Mac-1)
CD40: Member of TNF receptor family, expressed on B, monocyte and DC cells
TRANCE-R: TNF related activation induced cytokine-receptor
RANTES: Regulated on activation, normal T cell expressed and secreted
MIP1-α: Macrophage Inflammatory Protein -1α
MIP-1 β: Macrophage Inflammatory Protein -1β
LCs: Langerhans Cells
MiHA: Minor Histocompatibility Antigens
PCR: Polymerase Chain reaction
GALT: Gut-Associated Lymphoid Tissue
VCAMs: Adhesion molecule expressed on endothelial cells, binding to VLA-4 ligand
NCAMs: Adhesion receptor molecule in the nervous system
FACS: Fluorescence Activated Cell Sorter
GvDH: Graft versus Host Disease
DST: Donor Specific Transfusion
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CHAPTER 1
Chapter 1: INTRODUCTION

1.1 Preface:

A central problem in transplantation remains the immune response of the host against graft antigens (Ags) of the donor. T cells play a more dominant role in the rejection response than do B cells. One of the basic tenets of transplantation immunology is that tolerizing the T cell compartment to alloantigens will lead to long-term graft survival in unsensitized recipients. Immunologic tolerance can be operatively defined as a state in which the recipient’s immune system fails to react to specific foreign Ags, without signs of concomitant non-specific immunosuppression. Although the induction of transplantation tolerance has been achieved in some experimental animal models, few cases of true transplant tolerance have been described in humans. Instead, clinical transplantation routinely involves using non-specific immunosuppressive agents, which in turn leave patients susceptible to malignancy and opportunistic infections. In addition, these immunosuppressive reagents and/or their metabolites are often non-specifically toxic to the host/transplanted organ.

Given the side effects attributable to nonspecific immunosuppressive therapy, there is now a growing interest in exploring ways of achieving antigen-specific immunosuppression in adult animals in vivo. Amongst protocols which have been proposed for clinical use are: donor-specific pretransplant transfusion, believed to tolerize by virtue of presentation of donor Ag on inefficient antigen presenting cell (APC); and the use of nondepleting monoclonal antibodies (mAbs). Both techniques may lead to the induction of anergy, a state of lymphocyte unresponsiveness induced following ineffective signaling in cells after initial Ag encounter.

Ag specific tolerance induction has been shown by a number of laboratories, including our own, to follow portal venous immunization. In experimental model systems our laboratory has shown that portal venous (pv) pre- (or peri-) transplant immunization with donor Ag can produce graft specific
hyporesponsiveness, leading to delayed rejection of both vascularized and non-vascularized allografts. This delayed rejection is associated with a decreased production of distinct cytokines, IL-2 and IFN-γ, produced after Ag-specific restimulation in vitro of lymphocytes taken from the grafted animals. Concomitantly, there is an increased production of IL-4, IL-10, and IL-13 from activated T cells.

A suppressive subtractive hybridization approach was used to explore a role for other genes, which are differentially expressed after pv immunization. One of the products obtained encodes a molecule, OX-2, described previously as belonging to the immunoglobulin (Ig) supergene family. Preliminary data, relying on (an unexpected) cross-reactivity between a commercial antibody to rat OX-2 and the murine OX-2 molecule, suggested that expression of OX-2 was functionally relevant in the regulation of graft rejection following pv immunization. The purpose of the research project outlined in this thesis was to raise a monoclonal antibody to murine OX-2, and investigate whether this monoclonal antibody could reverse the tolerance induction developing in pv immunized animals.
Review of literature:

1.2 T cells and T cell subsets:

1.2.1 Effector function:

T cells have been divided into at least two subsets, depending on their mode of antigen recognition. CD4+ ("helper") T cells recognize antigen presented in association with Class II MHC molecules, while CD8+ ("cytotoxic"/"suppressor") T cells recognize antigen presented in association with class I MHC molecules (1). Note that T cell activation also requires concomitant delivery of so-called costimulatory signals by molecules (ligands/counter-ligands) expressed on the surface of the T cell and the antigen-presenting cell (APC). Foremost amongst these costimulatory molecules are the following pairs: CD80, CD86 and CD28/CTLA-4; CD40 and CD40Ligand (2, 3). The generation of proliferating CD4+ Th cells is believed to influence the development of the following Ag-specific effector mechanisms:

(i) Production of specific antibody (Ab) by B-cells:

Rejection is associated with a number of histopathological changes, including arteriolar thrombosis, interstitial hemorrhage and fibrinoid necrosis of the arteriolar walls. This was thought to be due to the deposition of antibody and fixation of complement. However, the appearance of donor-specific antibody does not necessarily precede rejection and its presence may be compatible with unimpaired graft function (4). Nevertheless, in experimental models in which donor and recipient rats differ only for MHC class I antigens, acute rejection of the kidney grafts seems to occur as a result of production of donor-specific antibody initiation (5). In general, for allografts, it is thought that T cell immunity is most relevant for rejection processes. However, in the case of xenografts (transplants between a species) antibody-mediated mechanisms of graft injury are clearly very important.
(ii) Specific cytotoxic T cells (CTLs):

Using in vitro systems, MHC mismatched lymphocytes proliferate and produce cytokines after allo-recognition, in a so-called mixed lymphocyte reaction (MLR). The resulting cytokine production contributes to the generation of cytotoxic T cells (CTLs) that specifically lyse target cells bearing the mismatched MHC antigens (6). It has been suggested that the MLR and resulting cytotoxicity is an in vitro correlate of graft rejection, with the proliferation representing the expansion of antigen specific cells following recognition of alloantigen, along with their subsequent cytokine production, and the CTLs the effector cells of graft rejection. There are a number of lines of evidence to suggest that CTLs may be involved in graft rejection. First, CTLs may be recovered from allografts that are undergoing rejection but they are present only at low levels in grafts of animals that have been treated with cyclosporine to prevent rejection (7). Second, it is clear from experiments with cloned populations of CTLs that such cells are capable of causing the type of tissue damage associated with rejection (8). Third, graft rejection may be delayed in the absence of CD8⁺ cells (9).

However, others have shown that graft destruction may occur in the absence of specific cytotoxic T cells and the presence of such cells within the graft may not always lead to graft destruction (10). Other experiments have demonstrated the presence of cytotoxic effector cells within a graft that is not rejected (11). These results imply that CTLs are neither necessary nor sufficient for graft rejection. It is also possible that the action of such cells may be blocked in situ or that the activity of cytotoxic cells in vitro does not accurately reflect their in vivo potential.

(iii) Delayed type hypersensitivity (DTH):

CD4⁺ T cells initiate the so-called DTH reaction (12) that is elicited by antigen encountered first at the skin surface. It is characterized by an infiltrate of lymphocytes and cells of the monocyte-macrophage lineage. Support for a role for DTH-type reactions in graft rejection came initially from studies on rejection of H-Y disparate grafts. Although cytotoxic cell responses did not correlate well with graft rejection, the generation of DTH reactions to H-Y antigens followed closely the ability of female
mice to reject male skin (12). Subsequently it was found that irradiated rats reconstituted with CD4 cells rejected heart grafts without developing a detectable specific cytotoxic response within the transplant (13). Data from experiments such as these suggest that DTH reactions are more important in the process of skin, and even cardiac graft rejection, than are cytotoxic T cell responses. It seems likely that neither mechanism is universally uniquely crucial for graft rejection.

1.2.2 T cell cytokines and the heterogeneity of T-cell populations:

Cytokines are thought to be important regulators of immune and inflammatory responses. Not only do they control the magnitude of such responses, but they also regulate the differentiation of effector cells thereby controlling the nature of the immune response. Discrete cytokines are centrally involved in the proliferation and differentiation of bone marrow cells and progenitors of each leukocyte subset.

Cytokines exist largely as soluble proteins, which act primarily at a local level. The evidence for the existence of subsets of CD4+ Th lymphocytes that differ in their cytokine secretion patterns and effector functions has provided a framework for understanding the heterogeneity of immune response. Mosmann et al. demonstrated that mouse CD4+ T-cell clones could be classified into distinct populations on the basis of their patterns of cytokine production (14). They termed the two populations Th1 and Th2. Th1 clones produced IL-2, IFN-γ and tumor-necrosis factor (TNF-β), whereas Th2 clones produced IL-4, IL-5, IL-6 and IL-13. Although IL-10 was originally described as a product of Th2 clone (15), it is now clear that IL-10 is also secreted by Th1 cells (especially in humans) (16) and by activated macrophages. Meanwhile, TNF-β has been shown to be produced by some Th2 cells (17).

There are other features of these clones that are important. First, it was shown that each T-cell subset produces cytokines that can serve as autocrine growth factors (18), promoting the differentiation of naïve T cells to that subset. Second, the two subsets produce cytokines that reciprocally regulate the development and activity of one another. For instance, IFN-γ produced by Th1 cells amplifies Th1 development and inhibits proliferation of Th2 cells (19), whereas IL-10 produced by Th2 blocks
activation of Th1 cells. The net result of cytokine-mediated self amplification and cross-regulation is that once a T-cell immune response begins to develop along one pathway, namely Th1 or Th2, it tends to become progressively polarized in one direction. **Fig 1.1** Shows CD4$^+$ T-cell activation programs (solid arrows indicate a positive effect, broken arrows indicate an inhibitory effect).

There are a number of studies, which support a physiological role for this dichotomous production of Th1/Th2 cells. First, studies of murine *Leishmania major* (*L. major*) infection showed, for the first time, that resistance and susceptibility to disease are attributable to specific Th1 and Th2 responses (20). Following infection with *L. major* susceptibility is associated with the early production of IL-4, while resistance is dependent upon IL-12 production. Interestingly, it has now become clear that this subdivision into type-1 vs type-2 cytokine producing cells can be extended to CD8$^+$ T cells (as well as CD4$^+$ T cells), so-called Tc1 and Tc2 (21).

### 1.2.3 Th1 and Th2 effector functions:

It has been argued that Th1 cells are primarily responsible for cell-mediated immunity or DTH, and Th2 cells for humoral immunity (21, 22). Supporting such a hypothesis, the principal Th1 effector cytokine, IFN-$\gamma$, has two key functions. First, it activates macrophages, enhancing their microbicidal actions. Second, IFN-$\gamma$ stimulates the production of IgG antibodies which bind to high-affinity Fc$\gamma$ receptors and are principal antibodies involved in the opsonization and phagocytosis of particulate microbes. IFN-$\gamma$-dependent isotypes are best defined in mice, and are mainly IgG2a and IgG3 (23). The human homologues are probably IgG1 and IgG3. It seems that one of the principal functions of Th1 cells is in phagocyte-mediated defense against infections.

The principal cytokines of Th2 cells are IL-4 and IL-5. IL-4 is the major inducer of B cell switching to IgE production and is therefore a key initiator of IgE-dependent, mast-cell-mediated reactions (24). IL-5 is a principal eosinophil-activating cytokine (25). Th2 cells are excellent helper T cells for B lymphocytes, and stimulate the production of high levels of IgM and non-complement-fixing
IgG isotypes, such as IgG1 in mice, or its homologue, IgG4, in humans (23). Several cytokines produced by Th2 cells have *anti-inflammatory* actions. IL-4 and IL-13 antagonize the macrophage-activating action of IFN-γ (14) whereas IL-10 suppresses numerous macrophage responses (26). Meanwhile, TGF-β (which is produced by some Th2 cells and many other cell types) is antiproliferative and inhibits leukocyte activation (27). Thus, the net result of Th2 activation is to inhibit acute and chronic inflammation, including DTH reactions.

**1.2.4 Development of helper T-cell subsets:**

Current thought is that Th1 and Th2 cells are not derived from distinct lineages which are committed before stimulation (28), but rather that both subsets develop from the same T-cell precursor, whose differentiation is influenced by the manner and environment in which those precursor cells are stimulated (28). The precursor is a mature, naïve CD4⁺ T lymphocyte that produces mainly IL-2 upon initial encounter with antigen. The most potent differentiation-inducing stimuli are cytokines themselves. IL-12, produced by activated macrophages and dendritic cells, is the principal Th1-inducing cytokine. At the transcriptional level, IL-12 activates three putative transcription factors, Stat1, Stat3 and Stat4 (29). In studies designed to elucidate which of these was most important for polarization in T cell differentiation it was found that Stat4 was activated only by IL-12, and that “knocking out” either IL-12 (30) or Stat4 (31, 32) resulted in markedly reduced Th1 responses.
Fig. 1.1 This figure adapted from Nickerson P. et al. 1997 (26)

Stimulating signal

Inhibitory signal
As discussed earlier, the development of Th2 cells from naïve precursors is facilitated by IL-4 (28), which signals through activation of Stat6 and a protein, first identified as an insulin-response substrate, called IRS-2 (33, 34). The mechanism by which Stat6 induces IL-4 production and Th2 development is unclear. However, consistent with an important role for IL-4 in Th2 development are the findings that knockout of the IL-4 gene (35, 36) or the Stat6 gene (37, 38) results in deficient Th2 responses, the converse of the IL-12 and Stat4 knockouts.

1.3 Antigen presentation and the development of immune responses:

1.3.1 Dendritic cells vs B cells and macrophages as antigen-presenting cells (APC):

T cells are stimulated by proteins which have been processed into peptides and presented in the context of MHC class I and class II molecules on the surface of APCs (39). The most potent APC described is the dendritic cell (DC). Antigen presented by DCs leads to efficient stimulation of both B and T lymphocytes (40). While both B cells and DCs are able to endocytose soluble proteins (41), it appears that macrophages are the most efficient at endocytosing large amounts of Ags (42), with DC up to 100 times more efficient than B cells (43). DCs are also able to take up particulate antigen for presentation on Class II MHC (44). In vitro studies have shown that antigen-pulsed DCs are the primary cells able to induce proliferation and IL-2 production in T cells (45). In vivo evidence also shows that DCs pulsed with Ags, but not antigen-pulsed macrophages or B cells, are able to initiate either a primary T cell response (44), or initiate humoral responses (46). More recently there is evidence that DC are important not merely in induction of immunity, but in tolerance induction also (47). Starzl et al (48) have suggested that migration of donor DC from a transplanted organ, into lymphoid tissue of the recipient, is essential for long-term graft survival, and that the survival of different organs is related to their DC content.
1.3.2 A role for immature antigen-capturing dendritic cells:

Despite the evidence for this key role for DCs in immune induction, in most tissues DCs are present in a so-called “immature” state, and are unable to stimulate T cells. It seems that these DCs lack requisite accessory signals for T-cells activation, including the molecules referred to as B7.1 (CD80) and B7.2 (CD86). Nevertheless, these immature DCs are well equipped to capture antigens. First, they can take up particles and microbes by phagocytosis (44, 49-51). Second, they can form large pinocytic vesicles in which extracellular fluid and solutes are sampled, a process called macropinocytosis (50). Thirdly, they express receptors that mediate adsorptive endocytosis, including C-type lectin receptors such as the macrophage mannose receptor (52) Fcγ and Fce receptors (53).

Once the DC has captured an Ag, a maturation signal is generated. The ability of the DC to capture Ags rapidly declines, and it continues the assembly and processing of Ag to form MHC peptide complexes. Mature DCs express high levels of the NF-kB family of transcriptional proteins (Rel A/p65, Rel B, Rel C, p50 and p52) (54) which regulate the expression of many genes encoding immune and inflammatory proteins. Signaling through the tumor-necrosis factor receptor (TNF-R) family, for example TNF-R (CD120a/b) or CD40 results in activation of NF-kB and the induction of the immune response through such activated DCs.

1.3.3 Migration of dendritic cells in vivo:

After activation, DCs travel to secondary lymphoid organs such as the spleen and lymph nodes. There, DCs complete their maturation (55), attract T and B cells by releasing chemokines (56) and maintain the viability of recirculating T lymphocytes (57) Amongst the chemokines produced by activated DCs are RANTES (Regulated on activation, normal T cell expressed and secreted), MIP1-α (Macrophage inflammatory protein -1α) and MIP-1β. These were the first chemokines for which lymphocyte - chemotactic activity was reported (58). More recently monocyte chemotactic proteins (MCP-1, 2,3 and 4) have also been found to be potent attractants of T lymphocytes and DCs (59).
In blood, there are two subsets of precursor DCs, one expressing and one lacking CD11c (60). Both subsets can enter lymphoid organs at high endothelial venules by virtue of interaction with the counter-ligand CD49d, β1 integrins (61), with different final destinations such as the B cell (CD11c+) and T cell (CD11c−) areas. Once activated by DCs, T cells interact with other cells, such as B cells for antibody formation, macrophages for cytokine release, and target cells for lysis (60).

MHC products and MHC-peptide complexes are expressed at 10-100 times higher levels on DCs than on other APCs including B cells and monocytes (62). Mature DCs are resistant to the suppressive effects of IL-10, but synthesize high levels of IL-12 (63-65), that has been shown to enhance both innate (natural killer cells) and acquired (B and T cells) immunity. DCs also express a number of accessory molecules that interact with receptors on T cells to enhance non-specific adhesion and promote cell signaling. Molecules functioning in this so-called co-stimulation capacity include, for example, LFA-3/CD58, ICAM-1/CD54, B7-1/CD80, B7-2/CD86 (66, 67). All of these properties (MHC expression, secretion of IL-12 and the expression of co-stimulatory molecules) are upregulated rapidly after maturation signaling in DCs.

The communication between DCs and T cells seems to be a bi-directional one, with DCs responding to T cell signals and vice-versa. Thus, CD40 (68) is ligated by the TNF family of proteins expressed on activated and memory T cells. This leads to increased DC survival (66), upregulation of CD80 and CD86 (68), secretion of IL-12 (63, 64) and release of chemokines such as IL-8, MIP-1α and β (68).

1.3.4 The potential role of dendritic cells in tolerance induction:

Increasing evidence implies a regulatory role for DC in tolerance induction. In the thymus, self-tolerance occurs by deletion of self-reactive T cells. Dendritic cells play a role by interacting with developing T cells in the thymic environment during negative selection. Experimental work in vivo has shown that intrathymic DC can present circulating soluble non-MHC antigen to T cells (69). Intravenous
injection of an enriched population of thymic DC pulsed with an encephalitogenic peptide of myelin basic protein (MBP) can prevent the development of experimental autoimmune encephalomyelitis. Protection is similar to that induced by direct intrathymic inoculation of MBP (70). This protection is lost in thymectomized recipients, emphasizing the important role of the thymic environment in the induction of tolerance. Intrathymic injection of Mls-incompatible spleen or thymic DC can also induce tolerance, this time apparently via clonal anergy induction (71). The ability of DC to induce tolerance is not restricted to thymic DC and a critical role for peripheral DC in central tolerance has been demonstrated by the restoration of tolerogenic properties of APC-depleted thymi using purified spleen DC (72).

Intravenous administration of antigen-pulsed Langerhans cell (LC) or spleen DC into syngeneic mice can also induce antigen-specific suppression of a DTH response which occurs after antigen challenge with the same antigen (73, 74). Coculture of bone marrow-derived DC progenitors with naïve T cells in vitro induced alloantigen-specific hyporesponsiveness, and systemic administration of these DC progenitors into allogeneic mice prolonged the survival of heart or pancreatic islet allografts in vivo (75, 76). The identification of other unique subsets of thymic and splenic DC, which express CD8 and induce apoptosis in activated T cells by Fas-FasL interaction, emphasizes the extensive phenotypic and functional heterogeneity among DCs in vivo (77, 78).

Multiple factors determine whether DCs induce tolerance or responsiveness. Finkelman et al. observed that DC can present Ag in either a tolerogenic or immunogenic fashion according to other manipulations. Thus the injection of mice with a rat-IgG2b anti-spleen DC antibody 33D1 induced rat IgG2b-specific T and B cell tolerance. In contrast, injection of mice with aggregated 33D1 plus IL-1 induces an IgG1 anti-rat IgG2b response rather than tolerance (79).

1.4 Regulation of T cell stimulation:

Maintaining homeostasis in the face of rapid and dynamic alterations in lymphocyte populations require effective mechanisms for preventing and terminating lymphocyte responses. These mechanisms
fall into two broad categories. First, the absence or loss of stimuli that provide necessary survival and growth signals to lymphocytes leads to a failure to initiate immune responses and functional inactivation or programmed death of lymphocytes. Second, lymphocyte activation itself triggers regulatory systems whose primary function is to control lymphocyte proliferation and differentiation.

For an immune response to occur, lymphocyte stimulation itself depends on exposure to two types of stimulus. The "first signal", an antigen, ensures the specificity of the response (80, 81). T cell activation starts with the ligation of an antigen-specific T cell receptor, TCR, by peptide contained within the groove of an MHC molecule. The TCR comprises two similar chains, the alpha and beta chains, which are complexed with several more chains of the CD3 complex. The TCR confers specificity of antigen/MHC binding whilst the CD3 complex transduces the activation signal to the T cell. Other signals important for T cell activation include those provided by costimulator molecules and cytokines which promote clonal expansion of the specific T cells and their differentiation into effector and memory cells.

The best defined costimulators for T cells are the two members of the B7 family, B7-1 (CD80) and B7-2 (CD86), which are induced on APCs by microbes and by cytokines produced during innate immune reactions. The counter receptors for B7.1/B7.2 are CD28 and CTLA-4, the latter potentially implicated in delivery of negative signals (2). The engagement of CD28 by B7 results in the expression in T cells of antiapoptotic proteins of the Bcl family, notably Bcl-xL (82), and the production of cytokines, such as IL-2 (83). Costimulation also, occurs via the CD40 molecule on APCs, which interacts with its ligand (CD40) on T cells (3). Figure 1.2 represents a diagrammatic representation of the two-signal model of lymphocyte activation.

*Failure of lymphocyte activation:*

The notion that lymphocyte activation requires both Ag and second signals, and will not occur when either is absent, has been exploited to develop strategies for preventing allograft rejection, by blocking costimulation at the time of organ transplantation in animals (84). Clinical trials of costimulator antagonists for blocking pathologic immune responses are currently under way. Ag recognition by
lymphocytes without secondary (costimulatory) signals leads to a state of functional unresponsiveness. This state was first demonstrated with cloned lines of CD 4+ helper T cells, in which antigen receptors were engaged without costimulation (85). T cell anergy may also be induced under conditions where adequate costimulation is available but where the Ag receptor signal is suboptimal. An example would be when a T cell encounters an altered peptide ligand, which does signal optimally via the T cell receptor (86).

**Active termination of T cell immune responses:**

A unique feature of lymphocytes is that their activation itself triggers feedback mechanisms that limit their proliferation and differentiation. Three such regulatory pathways have recently been identified in T cells.

1. CTLA-4-mediated T cell inhibition. A second T cell receptor for B7 molecules, called CTLA-4, was shown to function primarily to shut off T cell activation (87). CTLA-4 is induced on T cells after activation, and upon binding B7 it transduces signals that inhibit the transcription of IL-2 and the progression of T cells through the cell cycle (88). The biochemical basis of negative signaling by CTLA-4 is not clearly defined. It is known that CTLA-4 blocks signals transduced by CD28, suggesting that these two B7-recognizing molecules function as mutual antagonists (88).

2. Fas-mediated activation induced cell death. Activation of T cells also leads to coexpression of the death receptor, Fas (CD95), and its ligand, FasL, resulting in death of the same and neighboring cells (89, 90). In T cells, this pathway of apoptosis is induced by repeated activation, is potentiated by IL-2, and is not prevented by constitutive expression of Bcl-2 or Bcl-xL both molecules reportedly involved in blocking apoptosis (91). It is likely that Fas-mediated death of T lymphocytes is most important for eliminating cells that repeatedly encounter persistent Ags. This hypothesis is supported by the observation that mice with mutations in Fas or FasL develop autoimmune disease but do not exhibit abnormally prolonged responses to viruses or immunization with foreign Ags (92, 93). A somewhat related mechanism for regulating activation (and producing unresponsiveness) is called
death by neglect. Lymphocytes that are deprived of survival stimuli, such as costimulators and cytokines, lose expression of antiapoptotic proteins, mainly of the Bcl family, and die “by neglect” (89). This pathway of apoptosis is referred as “passive cell death”.

3- IL-2-mediated feedback regulation. IL-2 is the prototypical T cell growth factor and functions in an autocrine and paracrine manner to stimulate clonal expansion of antigen-stimulated lymphocytes and “bystander” cells. This role of IL-2 is so well established that it came as somewhat of a surprise to discover that targeted disruption of the IL-2 gene leads not to profound immunodeficiency but to uncontrolled accumulation of activated lymphocytes and manifestations of autoimmunity (94). How IL-2 functions to control T cell expansion is not yet fully resolved (95).

**Cytokine-mediated regulation:**

The stimulation of naïve T lymphocytes by antigen and second signals leads to their differentiation not only into effector cells whose function is to eliminate the Ag but also into regulatory cells. Such regulatory T cells may function by producing cytokines whose net effect is immunosuppressive. Examples of such cytokines are transforming growth factor-β1 (TGF-β1) (96), which inhibits lymphocyte proliferation, and IL-10, which inhibits macrophage activation and the expression of costimulators (97). These inhibitory cytokines limit the expansion of specific lymphocytes and return activated macrophages and other inflammatory cells to their normal resting state.
Signal 1 alone;
- Failure to activate
- T cell anergy (inability to respond on subsequent exposure to antigen)

Signal 1 + second or costimulatory signals;
- Full T cell activation

Fig. 1.2 Diagrammatic representation of the two-signal model of lymphocyte activation.
1.5 Tolerance:

Antigen-specific tolerance has been explained as resulting from the operation of a number of different mechanisms. Included in these are clonal deletion; stimulation of regulatory cells; development of anti-idiotypic antibodies; alteration in T-cell subsets; regulation of cytokines; ignorance; anergy and chimerism.

1.5.1 Clonal Deletion

Deletion of antigen reactive T cells is an important mechanism for the induction of tolerance to self-antigens in the thymus during the development of the immune system, so-called central tolerance (98). However, as a mechanism for the induction of peripheral tolerance it is believed to be less important. It has been identified as the mechanism responsible for the induction of tolerance to a superantigen, Mls1\(^{+}\), in thymectomized adult mice (99), although a similar series of experiments examining the mechanism of tolerance induction to the same superantigen in normal adult mice did not identify deletion as a mechanism (100). These two studies highlight the importance of other (unknown) factors in determining which mechanism is in operation.

1.5.2 Regulatory Suppressor and veto cells

CTLs, CD8\(^{+}\) T cells, recognize the MHC class I molecule (HLA-A, B, C in human) and its associated peptides (101). CTLs cause cellular death by making holes in the cellular membrane using a molecule called “perforin” or by inducing “apoptotic” cellular death. They constitute the major effector cell type of allograft rejection. CD8\(^{+}\) T cells were, somewhat paradoxically, also described to be suppressor cells and veto cells, inhibiting allogeneic immune responses. Veto cells are cells which, when recognized by CTL, are able to kill the CTL and hence “veto” their own cytolysis (102). Suppressor cells of recipient origin, and veto cells of donor/recipient origin, have both been implicated in the tolerogenic
effect of donor specific transfusion (DST) (103). Functional evidence for the existence of alloantigen suppressor cells of T cell responses has been obtained in several transplantation models (104) but such cells have been cloned in only a few instances and it has been difficult to identify suppressor cell specific markers. Few mechanisms of specific suppression of alloreactivity have been defined. One mechanism may involve production of the cytokine TGF-β, which is also believed to be involved in the veto cell-mediated inhibition of CTL activity (105).

1.5.3 Alteration of T-cell subsets and tolerance induction:

The central role of CD4+ cells and their participation in the effects of specific DST has been demonstrated in an experimental model in which a non-depleting anti-CD4 antibody was given pretransplantation with DST to induce tolerance (106). One mechanism by which this effect occurs may reflect an alteration in the respective expansion of T-helper 1 to T-helper 2 subsets by DST. In an extensive series of studies from our laboratory it has been shown that tolerance induction following pv immunization is correlated with an increase in type 2 cytokine production vs type 1 cytokine production (107-113). Similar findings have been made by other groups (114). However, this story is clearly not the "whole one" since data from a variety of knockout animals has shown, for instance, equivalent tolerance induction in IL-4 knockouts and graft rejection in IL-2 knockouts (115, 116).

1.5.4 Clonal ignorance:

Potentially active T cells may remain operationally tolerant by virtue of so-called ignorance of an antigen. This phenomenon was described in an experimental system in which mice were produced carrying as transgene a glycoprotein from the lymphocytic choriomeningitis virus (LCMV) (100). The transgene, which was expressed by pancreatic β cells, was apparently ignored by the immune system, but following specific immunization, LCMV-specific T cells destroyed the pancreatic β cells and the mice became diabetic.
Work from Miller and his colleagues has shown that in transgenic mice where H-2Kb is expressed predominantly by pancreatic β cells, high-affinity T cells are deleted in the thymus as a result of low-level expression of Kβ in the thymus (100). T cells that escape deletion cause rejection of skin grafts expression Kβ, but are seemingly unaware of Kβ expressed by β cells in the same mouse. The β cells are destroyed only when high levels of IL-2 are present.

1.5.5 Anergy and tolerance induction:

The encounter by T cells of peptide/MHC complexes in the absence of the necessary costimulatory signals may result in anergy (85). Once a cell is rendered "anergic", triggering via its TCR fails to induce optimal proliferation. The anergic state is associated with a lack of IL-2 production and can generally be overcome by providing exogenous IL-2 (83). In a transplantation model, where tolerance was induced by pretreatment with donor alloantigen, allo-specific T cells were found within the grafts of (tolerant) recipients (11), but these graft infiltrating cells were unable to respond to, or produce, IL-2 in vitro (100). These data were interpreted to suggest that the donor reactive cells within these grafts were anergic. Others have shown that mRNA for IL-2 was still detectable in the graft-infiltrating population, which implies that the defect in the IL-2 pathway is not at the level of gene transcription.

Many studies have shown that there is a difference between the pattern and kinetics of cytokine expression in rejecting and tolerant recipients (100). A role for T cell anergy induction has been studied in several animal models of allotransplantation (117). Induction of T cell unresponsiveness to TCR cross-linking has been described after transplantation of I-Eα islet allografts into I-Eα recipients along with anti-CD4 mAb treatment (117). It is possible that donor- specific lymphocyte transfusion facilitates tolerance induction (118) due to their content of T cells, which present Ag in that manner that induces anergy (119). Blocking costimulatory molecule pathways (CD80/CD86 and CD28, CTLA-4), or CD40:CD40Ligand, has been shown to produce tolerance in vivo, with prolonged graft survival (120, 121).
1.5.6 Chimerism and tolerance:

Donor haematopoietic cells have been detected in recipients following organ transplantation. Such recipients are described as exhibiting peripheral donor microchimerism (48). It is proposed that donor chimerism may play an important role in the effect of DST and maintenance of unresponsiveness. Earlier studies suggested that the DST regimen facilitated the induction of chimerism (122, 123). Nevertheless the hypothesis that chimerism itself is causally implicated in graft tolerance remains controversial (124).

1.5.7 Role of γδ TCR+ cells in tolerance:

During thymic development, immature T cells rearrange and express genes encoding the T-cell antigen receptor, maturing to either αβ or γδ lineage T cells. The role of γδT cells in immunity has remained an enigma (125). Our laboratory has shown that γδT cells are expanded in an oligoclonal fashion following pv immunization, and anti-γδ TCR mAbs block the graft prolongation (tolerance) afforded by pv immunization (126). In independent studies it has been shown that anti-γδTCR also blocked tolerance induction to ovalbumin (OVA) following oral immunization (127), and that oral administration of OVA did not induce tolerance in δ-chain knockout (δ-/−) mice (128, 129).

A number of observations implicate γδTCR+ cells in a variety of conditions associated with chronic stimulation/inflammation in vivo, including multiple sclerosis and arthritis (129-131). γδTCR+ cells can be activated to produce a number of cytokines as well as to develop non-MHC-restricted cytotoxicity (132). They can produce cytokines that alter the Ag-presenting function of LCs, epidermal APC for (αβ) T cells (133); produce either type 1 or type 2 cytokines in response to Nippostrongylus brasiliensis or Listeria monocytogenes infection (134); and are a source of immunoregulatory TGF-β in the uterus during pregnancy (135).
1.6 Specific immune responses in transplant rejection:

1.6.1 The role of T cells and other cells in the process of graft rejection:

Transplant rejection can be defined as an immune response that mediates injury and destruction of transplanted tissue. Immune responses relevant in graft destruction can be categorized as non-specific inflammatory responses and as antigen-specific immune responses. The specific immune response is mediated by thymus-derived T cells and antibody producing B cells. As noted at the outset, T cells do not recognize free native antigen, but rather recognize short peptides of a few amino acids in length derived from proteins which are presented on the surface of APC in associated with MHC molecules (136-138). In general, T cells bearing the CD4+ or CD8 +cell surface proteins recognize antigens in the context of MHC class II or class I respectively. The principal targets of the immune response to allografts are the MHC molecules themselves (139, 140) and T cell recognition of allo-MHC is the primary event that initiates allograft rejection.

Allo-MHC molecules induce uniquely strong primary immune responses in vitro in the mixed lymphocyte response (MLR) and in cytotoxic lymphocyte (CTL) assays (141, 142) and these reactions are hypothesized to be the in vitro equivalents of reactions producing rejection in vivo. It has long been recognized that the normal T cell repertoire contains a high frequency (1-10%) of total T cells capable of responding to allo-MHC molecules (143). Two conundrums for immunologists have been: “why is the frequency of alloreactive T cells so high” and, “how do positively selected, self-MHC-restricted, T cells recognize both foreign antigens and allo-MHC?” The answer to understanding these questions depends upon a more detailed analysis of T cell allo-recognition.

As discussed T cell receptor Ag recognition involves a tripartite structure consisting of T cells, MHC molecules, and a peptide bound in the groove of the MHC molecule (144). There are at least two distinct pathways of allo-recognition. In the so-called “direct” pathway, T cells recognize intact allo-MHC molecules on the surface of donor or stimulator cells. However, in the so-called “indirect” pathway, T cells recognize processed alloantigen in the context of self-APCs.
(i) T cell recognition of intact allo-MHC (the direct pathway):

In 1977, Matzinger and Bevan proposed that a single allo-MHC product can stimulate numerous T cell clones by forming multiple "binary complexes" with endogenous molecules. These complexes result from the association of native cell surface molecules with MHC molecules in the cell membrane (145). Bevan proposed that the specificity of alloreactive T cell clones is for the allo-MHC molecule itself, and thus all the foreign MHC molecules on allogeneic cells (high determinant density) could stimulate T cells to respond (146).

(ii) Self-restricted T cell recognition of processed alloantigen (the indirect pathway):

An alternative mode of allo-recognition, called the indirect pathway of allo-Ag presentation, exists as a mechanism for initiation and/or amplification of allograft rejection. In this case, donor alloantigens are shed from the graft, taken up by recipient APCs, and presented to T cells. Chicz et al. (147, 148) in humans and Hunt et al. (149) in the mouse demonstrated that at least some of the peptides eluted from recipient cell surface class II MHC molecules represented donor MHC sequences.

These studies suggested that processing of MHC by self-APCs may be a physiologic event in vivo. Allografts may thus shed fragments of allo-MHC that are then processed by host APCs and subsequently presented as allopeptides to T cells on self-MHC. This indirect pathway of allore cognition may lead to the activation of T helper (Th) cells, which secrete cytokines and provide the necessary signals for the growth and maturation of effector CTLs and B cells leading to allograft rejection (150-153).

(iii) CD8+ CTL mediated mechanisms of graft rejection:

A key factor in the process of graft rejection is played by CTLs. CTLs possess two major effector mechanisms for induction of lymphocyte-mediated cytotoxicity (154). The granule exocytosis pathway, involves the soluble mediators perforin and granzyme B (GrB), stored in cytoplasmic granules in the CTL. Upon binding of a CTL to a target cell, the cytoplasmic granules of the CTL fuse with the plasma
membrane and perforin and GrB are released into the intracellular cleft between the CTL and target cell. In the presence of extracellular Ca\(^{2+}\) ions, perforin inserts into the plasma membrane of the target cell and polymerizes to form transmembrane channels, which then facilitate target cell death by osmotic lysis. Perforin pores also promote entry of GrB into target cells, which then serves to engage the apoptotic machinery of the target cell through the activation of interleukin-1β-converting enzyme family proteases (ICE) (155). Studies have shown that there is significant increased expression of GrB by graft-infiltrating T lymphocytes during acute renal allograft rejection (156), while others have reported increased expression of perforin (157).

A second mechanism of CTL cytotoxicity involves Fas ligand (FasL), a cell surface molecule belonging to the tumor necrosis factor family. Fas (CD95), a cell surface molecule belonging to the tumor necrosis factor receptor family, serves as receptor to FasL (158). Increased intragraft expression of FasL has also been reported to be correlated with acute renal allograft rejection (159).

(iv) NK mediated cell cytotoxicity and graft rejection:

Recently, it has been proposed that CD4\(^+\) T cells may also co-opt the cytotoxic machinery of other effector cells such as natural killer cells (NKs) and macrophages in graft rejection processes (160). NK cells are a major effector component of innate immunity. These cells are able to kill autologous cells infected with intracellular pathogens, as well as tumor cells and non-self cells. Protection of self from NK cytotoxicity is dependent upon so-called killer-inhibitory receptors (KIR) on the surface of the NK cell, triggered by self (but not non-self) MHC (161).

NK cells are a major source of interferon-gamma (IFN-γ) in human PBMC. IFN-γ has been postulated to sensitize T cells to the effects of IL-2 (162), promote cell mediated immunity, and activate macrophages before the development of antigen-specific T cell responses (163). NKs cells are known to be involved in the rejection of allogeneic bone marrow grafts (164). However, in a variety of experimental models, it has been found that vascular (and non-vascular) grafts survive indefinitely in the
presence of high levels of NK effector activity (7). Thus while clearly a potent source of cytotoxic activity, a role for the NK cell in organ graft rejection remains to be firmly established.

1.6.2 The Th1/Th2 paradigm in the context of transplantation:

Since Th2 are inhibitory to the development and function of Th1 (165), it has been proposed that preferential induction of allograft-specific Th2 would inhibit Th1-regulated rejection responses, allowing for a reduction or elimination of conventional immunosuppression. Much of the data used to support this concept is indirect and cannot be used to imply a causal role for Th1/Th2 cells in rejection or protection from rejection respectively. Steiger et al. have used mice deficient in IL-2 to test whether IL-2 is a critical growth factor in islet graft rejection and whether in the absence of IL-2, but presence of IL-4, tolerance develops. In these knockout mice IL-2 is not required for islet graft rejection as all transplants are destroyed (116). The experiments further demonstrate that IL-4 expression in the absence of IL-2 does not result in tolerance. Their experiments also noted IFN-γ was strongly expressed in the grafts of IL-2-deficient mice alongside IL-4 and IL-10, suggesting that a shift towards a classical Th2 dominated response had not occurred.

Several reports indicate that Th2 do not completely inhibit Th1 responses after transplantation, and may in fact promote alternate forms of rejection. Lang et al. reported that during acute human liver allograft rejection, levels of IL-4 and IL-10 in bile were approximately 10-fold higher than those observed for IL-2 and IFN-γ, indicating that overexpression of Th2 cytokines is not protective (166). Intragraft Th2 cytokine gene expression was induced in a mouse vascularized cardiac transplant model by either depleting recipients of CD8+ T cells (167) or by treatment with IL-12 antagonists (168). In both settings, intragraft IFN-γ expression was not inhibited and grafts were still rejected, indicating that Th2 cytokines did not prevent in vivo Th1 development, and did not foster graft acceptance. However, Chen et al. (169) reported that induction of neonatal tolerance was dependent upon ablating Th1 development. Treatment with exogenous IFN-γ prevented tolerance despite the fact that IL-4 producing Th2 were still present.
Others have evaluated the ability of exogenous IL-10, which inhibits Th1 cytokine production in vitro, to promote graft survival. Contrary to what might be expected, Qian et al. (170) found that posttransplant treatment with mouse IL-10 accelerated cardiac allograft rejection. Similarly, Zheng et al. (171) treated islet cell allograft recipients with a noncytolytic IL-10-immunoglobulin fusion protein, which has an enhanced in vivo half-life relative to native IL-10. Treatment with this IL-10 fusion protein tended to accelerate rejection rather than prolong graft survival, and also increased expression of the activated CTL product, granzyme B, in the regional lymph nodes. In contrast, Qin et al. reported that retroviral-mediated gene transfer of viral (v) IL-10, which lacks several of the proinflammatory activities of cellular IL-10 (172), prolonged graft survival in the nonvascularized neonatal cardiac transplant model (97). In agreement with these studies, Martinez and Krams reported an association between human renal (173) and liver (174) allograft rejection and the intragraft production of Th2 cytokines. In these studies, IL-5, rather than IL-2 or IFN-\(\gamma\), was increased during rejection episodes.

In separate studies, cardiac allograft recipients were treated with either neutralizing anti-IL-12 antibodies or homodimers of the IL-12 p40 subunit, which bind one component of the IL-12 receptor (IL-12R) and serve as a competitive inhibitor of bioactive IL-12 p70 (175). Although treatment with anti-IL-12 antibodies or p40 homodimers induced Th2 cytokine expression, graft rejection was accelerated rather than inhibited. Further, IL-12 antagonism failed to inhibit IFN-\(\gamma\) gene expression or in vivo Th1 development, indicating that IL-12 is not a pre-requisite for alloreactive Th1 (176).

In contrast to the above, studies in our laboratory have shown that skin allograft survival in mice is increased in an Ag-specific fashion if animals receive Ag-specific pre- or peritransplant pv immunization (108, 109). This specific graft enhancement was associated with the preferential activation of Th2 cells in these mice, with enhanced production of IL-4, IL-10 and a simultaneous decrease in the production of IL-2 and IFN-\(\gamma\). Mice receiving specific immunization via the lateral tail vein (intravenously; iv) do not show this enhancement or the qualitative change in cytokine profiles. In a modification of this approach, our laboratory showed an enhanced graft survival (with diminished IFN-
γ/IL-2 and enhanced IL-4/IL-10 production) in mice given B10.BR cells iv followed by injection with anti-IFN-γ antibody every second day (110). Furthermore, anti IL-10 monoclonal antibodies (but not anti IL-4 or anti IFN-γ) blocked graft enhancement after pv immunization (177). Donckier et al. also reported that neonatal mice given semi-allogeneic cells as a tolerizing regimen and treated with anti-IL-4 mAb during the inductive phase showed reversal of tolerance, implying a critical role for IL-4 in this process of tolerance induction (178). Interestingly, Qin et al. have found that retroviral transfection of cardiac grafts with viral IL-10, rather than murine IL-10, led to prolonged but not indefinite cardiac allograft survival, suggesting that the structural differences in IL-10 (viral/cellular) might determine whether the immunosuppressive versus immunostimulatory effects of IL-10 are seen (179).

Several immunosuppressive regimens that promote adult allograft survival appear to inhibit Th1 yet spare Th2 cytokine production (109, 180-182). These observations support a hypothesis that “graft accommodation”, which occurs in some individuals with time after transplantation, results in part from an immunosuppressive drug-induced shift from a Th1 to a Th2 dominated response. Interestingly Gajewski et al. reported that Th1 clones were more susceptible to Cyclosporine A (CsA) suppression than were Th2 clones (183), and Ramirez et al. found that the presence of glucocorticoids during primary stimulation of CD4+ T cells promoted expression of Th2, but not Th1 mRNAs upon restimulation of these cells (184). Sayegh et al. reported that blocking the CD28-B7 T cell costimulatory pathway promoted long-term acceptance of rat renal allografts, and was associated with inhibition of intragraft IL-2 and IFN-γ, but not IL-4 and IL-10, as assessed by immunohistochemistry (180).

It should be clear from these analyses that there is no simple resolution of the role of cytokines (type-1 vs type-2) in graft rejection/survival. Potential confounding variables include the animal systems studied, the nature of the graft (vascular/non-vascular) and or graft organ, auxiliary immunosuppression etc. Extrapolation from these animal studies to man is even more problematic.
1.6.3 Costimulation and the B7 family of proteins in transplant rejection:

Kuchroo et al., and Freeman et al., have suggested that although B7.1 induces a Th1 dominated response, B7.2 may induce Th2 cells (176, 185). The beneficial effect of the B7.1 antibody can be abrogated through the simultaneous administration of neutralizing IL-4 antibody with B7.1 antibody, suggesting that blocking the B7.1 signal passively allows the development of an IL-4 dominated Th2 response. In the transplantation setting, one might imagine that use of B7.1 antibodies would be beneficial if a Th2 response were involved in prolonged allograft survival. However, others have shown that anti-B7.2 antibodies but not anti-B7.1 have a beneficial effect on graft survival. Thus Lenschow et al., in studies of the non-obese diabetic (NOD) mouse (a model for insulin-dependent diabetes mellitus (IDDM)), showed that anti-B7.2 but not -B7.1 antibodies prolonged graft survival of allogeneic islet grafts (186). It should be acknowledged, however, that the effects of B7 blockade are complex. How signaling from B7-1, B7-2 (and CD40: CD40Ligand), with their counter-ligands CD28/CTLA-4 are integrated for T cell signaling remains an enigma.

1.6.4 The effect of donor-specific transfusion as a strategy for immunomodulation and development of tolerance induction:

DST has been used historically in human organ transplantation (187, 188). However, with the concern that DST may sensitize patients, thereby preventing transplantation, and with the introduction of the more effective non-specific immunosuppressive agent cyclosporine, the use of DST was curtailed. It has been demonstrated that antigen administered into the portal vein may be tolerogenic (189, 190). Pv, but not iv administration of allogeneic lymphoid cells induced allospecific tolerance, as manifested by abrogated DTH response to donor antigen (189). In addition, donor lymphocyte antigen administered to rats via the portal vein, but not systematically, resulted in prolonged survival of heart (191) and kidney grafts (192), while Rao et al. reported that systemic DST also prolonged subsequent renal allograft
survival (193). Possible explanations for these conflicting results may be attributed to differences in timing, dose, and the strain combinations used in the different studies (194).

As discussed above, previous work from our laboratory has shown that Ag-specific delayed graft rejection in vivo, and decreased allostimulation in vitro occurs after donor-specific pv immunization (107-109). These functional changes are associated with preferential activation of Th2 type cytokines rather than Th1 cells (110), in recipient animals. Similar changes were seen in rat small bowel or renal allografts (111), mouse renal allografts (195) and in human liver graft recipients (112). We argued that the changes observed were a function of a unique mode of Ag presentation which occur within the hepatic environment (113).

The mechanisms, by which polarization of cytokine production from T cells stimulated in vivo occur, are still unclear. As explained above, an imbalance in any one (type 1 or 2) cytokine alone is probably insufficient to explain transplant rejection or graft acceptance. Some of the controversy in this area may relate to the different allograft models studied (MHC compatible/incompatible, different tissues grafted, etc.) and the variable importance of different cells (CD4+, CD8+, macrophages, and Ig-producing cells) in those models.

In order to address the question of why pv immunized animals accepted grafts longer than iv immunized animals, we investigated differentially expressed genes in the two groups. A powerful mechanism for identifying and isolating cDNAs of differentially expressed genes involves a subtractive hybridization approach. Using such an approach, cDNA from one population ("tester") is hybridized to excess mRNA (cDNA) of another population ("driver"). The non-hybridized fraction ("the target") is then separated by hydroxylapatite (196), avidin-biotin (197) or oligo-(dT)30-latex beads (198). A newer technique described by Diatchenko uses a PCR-based subtraction hybridization method in which amplification of "non target" DNAs in the PCR reaction is suppressed by attaching long inverted terminal repeats to the "tester" cDNA (199) (see, Figure 1.3). This technique was used in our laboratory to analyze differentially expressed genes in mice receiving allogeneic renal grafts along with peri-transplant donor-specific immunization. Differential expression of mRNAs for certain cytokines (as expected) and
other novel transcripts were detected. *One of these encodes a molecule, OX-2, characterized previously as belonging to the Ig supergene family.*

1.6.5 MRC-OX-2 and its importance in our model:

The MRC OX-2 antigen in rat was initially described as a 41KDA-47KDA glycoprotein that is expressed on the cell surface of thymocytes, follicular dendritic cells, B cells and neuronal cells (200, 201). Differences in the apparent size of this molecule in different tissues is probably a function of differential glycosylation (202). The rat counterpart has recently been described to have significant homology with members of the murine B7 family, showing >60% homology with murine B-7-I/B7-2 and <40% homology with the corresponding ligands CD28, CTLA4 (195). In preliminary reports from our laboratory we showed that an antibody to the rat OX-2 molecule abolishes renal graft prolongation in mice following pv donor-specific immunization and reverses the polarization in cytokine production seen after specific restimulation (195). By Flow Cytometry (FACS) analysis, using dual staining of hepatic mononuclear cells with anti-OX-2 and anti-dendritic cell monoclonal antibodies, increased numbers (4 fold) of OX-2+ dendritic cells were seen following pv immunization.

Borriello et al. recently reported that CHO cells transduced to express OX-2, unlike cells expressing B7-1, did not stimulate detectable levels of IL-2, IL-4 or IFN-γ from murine T cells. These OX-2 transfectants did not bind the soluble receptor reagents of B7/CD 28 pathway (CD 28-Ig and CTLA4 Ig)(203) We have suggested that OX-2 is a molecule that acts through a "non"-B7/CD28 pathway, leading to functionally distinct consequences, as reflected by the resulting cytokine profile (195, 203).

A criticism of the data we have reported on the functional activities of anti-OX-2 in reversing the effects of pv immunization (on graft survival/cytokine production) has been that we relied on a (putative) cross-reactivity of murine OX-2 and rat OX-2 for the anti-rat OX-2 antibody. Accordingly, it was
important to investigate the role of OX-2 in the regulation of transplant rejection using species-specific monoclonal antibodies.
**Hypothesis:**

It is proposed that OX-2 is a functionally important molecule in the increased graft survival seen after pv immunization. One hypothesis suggests that OX-2 delivers a tolerizing (negative) signal to T-cells which can be assessed by following alterations in cytokine production. The purpose of the work described in this thesis was to characterize, and explore the function in graft rejection of, a newly developed anti-mouse OX-2 monoclonal antibody.

**Specific Aims:**

1. Preparation and testing of monoclonal antibodies (mAbs) to murine OX-2.

2. To confirm that anti-murine OX-2 mAb blocks increased graft survival in mice following pv immunization, and to assess the time post transplantation at which anti-OX-2 produces this effect.
Fig. 1.3 This figure adapted from Clontech subtractive hybridization manual.

- pv immunized animal
  - PV
  - Poly (A+) RNA
  - Synthesis of cDNA using oligo (dT) as a primer
  - Tester
  - with adaptor 1
  - with adaptor 2
  - cDNA: cDNA hybrids
    - a
    - b
    - c
    - d
  - e
  - add primers + amplify by PCR
  - exponential amplification for (e) n+......x

- IV immunized animal
  - IV
  - Poly (A+) RNA
  - Driver (in excess)
  - ...... First hybridization
  - ...... Second hybridization
  - Fill ends
CHAPTER 2

NOTE: An abridged version of this chapter has been accepted for publication in the Journal “Immunology Letters”, as an original paper with the title:

“Preparation and functional properties of monoclonal antibodies to human, mouse and rat OX-2”.

Preparation and functional properties of monoclonal antibodies to mouse OX-2

2.1 ABSTRACT

We have prepared mouse hybridomas to a 43KDa molecule expressed in the thymus, on a subpopulation of dendritic cells, and in the brain, in tissue derived from mouse cells. Using CHO cells stably transfected with pBK vector(s) expressing a cDNA construct for the murine OX-2 gene, we show these monoclonal antibodies (mAbs) detect a molecule encoded by this mouse OX-2 construct (mOX-2).

Previous studies have implied that this molecule might serve an important role in regulation of cell signaling for cytokine production. Using one-way mixed leukocyte reactions we show below that when cells are cultured in the presence of the mouse-specific mAb, cytokine production becomes polarized "away from" type-2 cytokine production, with preferential increased expression of type-1 cytokine production. Furthermore, increased skin graft survival in mice receiving allogeneic skin grafts after donor-specific pv immunization is reversed by these mAbs, even when these antibodies are given several days after transplantation.
2.2 INTRODUCTION

In a previous publication we described use of a suppression subtractive hybridization approach to analyze differentially expressed genes in mice receiving portal vein donor-specific immunization as a means of promoting renal allograft survival (204). In addition to detecting differential expression of cytokine mRNAs, other novel transcripts were detected. One of these encoded a molecule, OX-2, previously described as being expressed on dendritic cells (200) and as having some homology with members of the B7 family (203). We reported that an antibody to rat OX-2 abolished graft prolongation following pv donor-specific immunization in a mouse model, and reversed the polarization to type-2 cytokine production seen in these animals after specific restimulation (195). It was somewhat surprising that this antibody, made in mice to an antigen expressed in rats, would recognize determinants on the murine homologue (205).

We report data below on the independent preparation of monoclonal antibodies (mAbs) to the mouse OX-2 molecules, using a crude 43KDa cell membrane antigen prepared from fresh spleen dendritic cells for immunization. After initial screening by ELISA, mAbs were further tested for detection of an antigen (Western analysis) in fresh brain extracts, and using CHO cells stably transfected with pBK vectors expressing cDNAs for the relevant species-specific molecule. Our data confirm that these mAbs can alter cytokine production after allogeneic stimulation, and can reverse the increased graft survival seen following pv immunization.
2.3 Materials and methods

Mice. C3H/HEJ (H-2b) and C57Bl/6. (H-2b) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were housed five cage and allowed food and water. All mice were used at 8-12 weeks of age.

Monoclonal antibodies (mAbs). The following mAbs from PharMingen (San Diego, CA) were used: anti-IL-2 (JES6-1A12; biotinylated JES6-5H4); anti-IL-4 (11B11; biotinylated BVD6-24G2); anti-IFN-γ (R4-6A2; biotinylated XMG1.2); anti-10 (JES5-2A5; biotinylated SXC-1); and mouse IgG1 isotype control (clone 107.3, BALB/c anti-TNP). Strepavidin horseradish peroxidase and recombinant mouse granulocyte macrophage-colony stimulating factor (GM-CSF) was also purchased from PharMingen.

NLDC-145 (anti-mouse dendritic cells), F (ab’) 2 rabbit anti-rat IgG rat IgG mouse IgG), and F (ab’) 2 rabbit anti-mouse IgG phycoerythrin (PE) were obtained from Serotec. Toronto, Canada.

2.3.1 Antigen preparation from cells and tissues

Spleen, Peyer’s Patch and mesenteric lymph node cell suspensions were prepared aseptically from individual mice of the different treatment groups in each experiment. The following technique was used when dendritic cells were obtained by culture of bone marrow cells in vitro (206). Bone marrow plugs were aspirated from the femurs of donor male C57Bl/6 (or BALB/c) mice, washed and resuspended in αF10. Cells were treated sequentially with a mixture of antibodies (L3T4, anti-thyl.2, anti-Ly2.2) and rabbit complement and dead cells were removed by centrifugation over mouse lymphopaque (Cedarlane Labs). Cells were washed three times in αF10 and cultured in 10 ml of αF10 in tissue culture flasks, at a concentration of 2x10⁶/ml with 500 U/ml recombinant murine GM-CSF (PharMingen). Fresh GM-CSF was added at 36-hr intervals. Cells were separated over lymphopaque on days 3.5 and 7 of culture, again reculturing in αF10 with recombinant GM-CSF. At 10 days, an aliquot of the sample was stained with DEC205, FITC anti- rat IgG, anti-OX-2, PE anti - mouse IgG, and FITC- anti- B7.1 or FITC anti- B7.2.
Mean staining with these antibodies in our hands using cells harvested from such cultures has been 93%±7%, 14%±5%, 78%±9% and 27%±6%, respectively. Remaining cells were washed and injected into the portal vein as described.

Fresh dendritic cells from spleen were isolated as non-adherent cells from overnight culture after initial adherence to culture flasks at 37°C for 90 min (206). Routine staining of mouse splenocytes with DEC205 and FITC anti-rat IgG, or FITC-MAC-1 before and after overnight incubation produced the following staining pattern in these adherent cells: 8%±2%, 90%±11% and 92%±9%, 9%±3% respectively. A crude (non-adherent) dendritic cell preparation was extracted with lysis buffer, titred to a protein concentration of 10mg/ml, and used for immunization. Some of the same material was used subsequently for antigen preparation for screening in ELISA assays.

When brain or thymus tissue were used in Western gel analysis, whole tissue was homogenized and extracted with lysis buffer containing 100% NP40 (207). The cells or homogenized tissue were scraped in 1ml lysis buffer containing a broad range protease inhibitor cocktail (10mg/ml leupeptin, 10mg/ml aprotinin and 1mM phenylmethylsulfonyl fluoride [PMSF]) and 100% NP40. Cell homogenates were boiled for 5 min in Laemmli sample buffer (50 mM Tris-HCl, pH 6.8; 10% glycerol, 2%SDS, 5% 2-β- mercaptoethanol, 0.05%bromophenol blue).

**2.3.2 Immunization and production of mAbs:**

Two Fisher 344 rats were immunized with mouse dendritic-cell derived antigen in Freund’s adjuvant. Three subsequent boosts were administered spaced at 3-week intervals. When the serum titre had risen more than 10 fold from a pre-immune serum sample, as determined by ELISA, the rats were boosted intravenously. Three days later, rats were sacrificed and spleen cells were harvested and pooled. Fusion of the splenocytes with YB2/0 parental myloma cells and selection in HAT medium was performed as previously described (208).
2.3.3 Protein determination

The protein concentration in all samples for immunoblotting and ELISA assays was determined by the Biorad detergent compatible (DC) protein microassay. Standard curves were made in each experiment by using 5 dilutions of the protein standard containing 0.2ug/ul to 2 ug/ul protein. The standard curve remained linear over this range of protein concentrations. The optical density of the standards and samples was measured in triplicates at wavelength 595nm (209).

2.3.4 Electroelution of OX-2 antigen:

SDS-PAGE gels containing electrophoresed protein were stained with Coomassie Brilliant Blue (Bio-Rad, Canada). The gel band of interest was excised and the stained protein was electrophoretically eluted from the minced gel. This partial purification of OX-2 protein was based on the previously reported molecular weight (M.Wt.) and the net charge of the rat protein. The pore size of the membrane used was 12.000-14.000 KDa (Spectra/Por, USA). The purification of OX-2 was performed at a constant voltage (50 V) for 12-16 hr (210).

2.3.5 Western immunoblot analysis

Equal amounts of protein from total cell lysates (15 ug) were separated by 12.5% SDS-PAGE (Polyacrylamide gel electrophoresis, 12.5% separation and 3.5% stacking gel) at 100 V for 2 hours, and blotted onto a nitrocellulose of 0.45 um (Bio-rad, Canada) for 100 V/2hrs. or overnight at 40V using a Bio-Rad Transblot apparatus. For the electrophoresis and protein transfer, a standard running buffer (25 mM Tris-Base, pH 8.3, 192 mM glycine, 20% [vol./vol.] methanol) was prepared. The membrane was blocked for 2 hr. in Tris-buffered saline (TTBS), pH 7.6 containing tween-20 (TTBS: 10mM Tris HCl, 150 mM NaCl, 0.05% [vol./vol.] tween-20), 2% skim dry milk at RT. For washing, the membrane was soaked in TTBS for 15 min. This was repeated twice for 5 min with fresh TTBS. Mouse IgG anti rat OX-
2 was used at an optimum dilution (1:500) as a positive control; the unknown rat IgG anti mouse OX-2 mAbs were used as test and incubated for 2 hr.

Following washing, second antibodies were added (goat anti-mouse IgG conjugated with horse radish peroxidase (HRP), (1:500) (Amersham Life Science, Canada) or goat anti rat IgG-HRP (1:500) (Bethyl Laboratories Inc.). Incubation was for 2 hrs. with all antibodies diluted in TTBS containing 2% skim milk powder (211). This was followed by washing for 15 min then 3-4 times for 5 min. A chemiluminescence kit (Amersham life science, Canada) and Kodak X-omat AR film (Eastman Kodak, Rochester, NY) was used for development and visualization of immunoreactive bands according to the manufacturer's instructions. Several film exposures of each blot were performed in order to assure that resultant signals were within the linear response range of the film. To determine uniformity of loading and transfer, nitrocellulose membranes were stained with Coomassie Brilliant Blue. The OX-2 bands were identified by molecular weight markers (Bio-Rad, Canada).

2.3.6 Indirect ELISA for specific antibody detection (mouse anti-rat OX-2)

Semi-purified OX-2 protein (Ag) (0.01-0.1 ng) from the electroeluted gels (above) was added to poly-L-lysine 100 ng/ml) coated wells of ELISA plates. Incubation was performed overnight to coat the plate with Ag at 4°C to prevent evaporation. Blocking used 200 ul of blocking reagent, prepared as a mixture of bovine serum albumin (BSA) 0.25 gm and 50 ul Tween-20 dissolved in 100 ml PBS (phosphate buffer saline). Incubation for 1 hr at room temperature prevented non specific binding. This was followed by washing once with PBS and twice with dH2O. First antibodies (the rat hybridoma supernatants to be tested and the positive control, a mouse anti rat OX-2) were added at the optimum dilution (found to be 1:500). As a negative control, normal rat (pre-immune) serum was added at a similar dilution. After the addition of the primary antibody in the appropriate wells (test, positive or negative control), incubation was performed for 2 hrs. at RT. This was followed by washing as in the preceding step. The second antibody, an anti rat IgG-ALP (Sigma Immunochemical, USA), (optimum dilution
was added to all the wells of the plate for the test and normal rat Ig control wells; anti mouse IgG-ALP was used for the positive control well. Incubation was again for 2 hrs, followed by the washing step described before. The plate was developed by the addition of substrate; p-nitrophenyl (P) (Bio-rad. Canada), at 37°C for 30 min. (212) and the reaction stopped by adding 0.5 M NaOH. Absorbance was measured in an ELISA reader at 405 nm. (Bio-rad, Canada). OD readings in control wells (no first antibody) were <0.020 in all cases.

2.3.7 Polymerase Chain Reaction (PCR)

PCR was used to amplify an 858 bp region of the OX-2 open reading frame (ORF) cDNA. Two single-stranded oligonucleotide primers (5’ and 3’ primers) were designed complementary to sequences flanking the segment of DNA (858bp) to be amplified. The 5’ primer was 36 bases long (58.3% G-C content) and included a BamH1 restriction enzyme site (GGATCC), kozak translational start sequence (GCCGCCACC) and initiation codon sequence (ATG). The 3’ primer was 27 bases long (25.9% G-C content) including a XbaI restriction enzyme site (TCTAGA) and the termination codon TTA. Cycling conditions using Taq DNA polymerase were 94°C for 4 min., followed by 35 cycles of 94°C for 45 sec., 58°C for 45 sec., 72°C for 3 min., followed by one extension at 72°C for 5min. PCR products were separated using a 1.5% agarose mini-gel in a horizontal electrophoresis apparatus (Bio-Rad, Canada).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sense/Identify</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>OP1 (+)</td>
<td>5' sense</td>
<td>5'- ATA GGATCC GCCGCCACC ATGGGCAGTCCGTATTCAGGAGA-3'</td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
<td>Kozak</td>
</tr>
<tr>
<td></td>
<td>initiation codon</td>
<td></td>
</tr>
<tr>
<td>OP2 (-) antisense</td>
<td>5' antisense</td>
<td>5'-ATC TCTAGA TTATTTCACTCTTTATGCTCATCCCATGTCAG-3'</td>
</tr>
<tr>
<td></td>
<td>XbaI</td>
<td>termination codon</td>
</tr>
</tbody>
</table>

**TABLE 2.1** Primers OP1 and OP2 were used to amplify the OX-2 cDNA from mouse cDNA(203).
2.3.8 A directional cloning strategy:

Both primers used in the PCR reaction had restriction sites synthesized at their 5' ends to facilitate directional cloning into a pBK eukaryotic expression vector. The PCR products were extracted from the agarose gel (1.5%) using QIAEX (QIAGEN, USA). All non nucleic acid impurities such as agarose, proteins, salts and ethidium bromide were removed during the washing steps. The pure DNA was eluted in 20ul dH2O. The pBK phagemid vector used (pBK-CMV) is a cloning vector of 4518 bp derived from a high-GPY number COLE1-based plasmid. This vector allows expression in both eukaryotic and prokaryotic system. Stable clone selection in eukaryotic cells is possible using G418 because of the presence of the neomycin and kanamycin resistance gene, which is driven by the SV40 early promoter with thymidine Kinase (TK) transcription, termination and polyadenylation signals. In pBK-CMV, prokaryotic expression is driven by a Lac promoter, which is repressed in the presence of the LacI protein. In bacteria expressing the Lac Z M15 mutation and LacI, colonies containing vector without insert will be blue in the presence of 5-bromo-4chloro-3-indoyl-B-D-galactopyranoside (X-gal). kanamycin-resistant colonies containing vector with the insert are white. This vector has a polylinker of 17 unique restriction enzyme recognition sites.

The restriction enzymes BamH1 and Xab1 were used to produce cohesive ends and to linearize the plasmid DNA. The digestion step was checked by running both the digested and undigested (circular) vector DNA along with a 1Kb marker on 1.5% agarose gel. The purified PCR product of 858bps for OX-2, containing both restriction sites (BamH1 and Xab1), was ligated to the linearized pBK-CMV vector using the complementary cohesive (sticky) ends. The ligation reaction used the 5X ligase buffer and T4 DNA ligase (GIBCO, Inc.). The in-frame sequence of the cloned cDNA was confirmed by dideoxy sequencing reaction (termination method) using T7 DNA polymerase (Pharmacia Biotech, Canada).
2.3.9 Transformation and Transfection:

Transformation was performed using electroporation with high voltage electric shocks to stably introduce the pBK-CMV containing the OX-2-cDNA insert into DH10B E.Coli cells. 20 ul of DH10B cells were transferred to the electroporation cuvette along with 1ul of the ligation mixture precipitated by 5M NaOAC and 100% ethanol. Shocked cells were added to LB-broth-kanamycin and shaken at 37°C (225-250-cycle/1m.) overnight. Cells were plated onto LB-agar containing both kanamycin and X-gal for selecting white colonies. The final cDNA template (cloned cDNA) was prepared from (3ml) overnight cultures of E.Coli in LB (Luria-Bertani) medium by using “Mini-Prep” plasmid DNA kits (QIAGEN, USA).

CHO cells were plated into a 24-well plate at 5x10⁵/well on the day before transfection. CHO cell transfection was conducted by superfect reagent (Qiagen-Canada). DNA (5ug) was diluted in TE, pH 7.4 (minimum DNA concentration: 0.1ug/ul) with cell growth medium containing no serum, proteins or antibiotics to a total volume of 150ul. The superfect reagent (20ul) was added to the DNA solution, vortexed and incubated for 5-10 min at room temperature to allow complex formation. The transfection complexes were added drop-wise onto the CHO cells in 60-mm dishes and incubated for 24-48hr at 37°C in 5% CO2. The medium contained remaining complexes were removed from cells by centrifugation. The cell pellet was resuspended in fresh medium and incubated for 24-48hrs in G418 selection medium. OX-2 expression by CHO cells was tested by Sigma Fast DAB (3,3’-Diaminobenzidine), the immunohistology substrate, which detects peroxidase activity (Sigma, USA). CHO cells were first stained with an unknown rat IgG anti mouse OX-2 mAbs ( in the studies below, data for clone 3.6 are shown) as a primary antibody for 2hrs. Following washing, the secondary antibody, goat anti- rat IgG conjugated with horseradish peroxidase (HRP) (1:1000) was added and incubation was for 2hrs. This was followed staining step with Sigma Fast DAB for 30 min.
2.3.10 FACS analysis of putative mAbs:

FACS analysis was performed using putative anti-OX-2 mAbs and the following cells. Fresh PBL lymphocytes (harvested after separation over mouse lymphopaque); spleen dendritic cells (isolated after adherence and overnight incubation, as above); and CHO cells transduced with either a pBK vector containing OX-2 or with control vector alone. FITC anti-mouse (or anti-rat) IgG was used as secondary antibody.

2.3.11 Mixed leukocyte reactivity (MLR) and cytokine production:

Allogeneic MLR cultures, using 1:1 mixtures of 2.5x10⁶ responder cells and mitomycin C treated stimulator cells, were set up in 24-well culture plates in 1ml of αMEM medium supplemented with 10 % FCS. Cells were obtained from C3H responder mice (with stimulator C57Bl/6). Culture supernatants were harvested at 60hrs and tested for different cytokines using previously described ELISA assays (mouse), or using CTLL-2 as bioassay for IL-2 production from all responder cell sources (213).

2.3.12 Pv immunization and skin allografts:

These techniques closely followed those described elsewhere (113, 126). In brief, C3H mice received pv immunization with 10x10⁶ C57BL/6 bone marrow derived dendritic cells (10 day cultures of C57BL/6 bone marrow in GMCSF and IL-4). Control groups received iv immunization or saline only. 3 days later all mice received C57BL/6 skin grafts. In some experiments (see text) mice also received iv infusion with 100μg/mouse anti-OX-2. Graft survival was followed daily.
2.4 Results:

2.4.1 Evaluation of a number of mAbs for staining of cell populations in fresh PBL or spleen:

All mAbs tested in the experiments described below were previously screened by ELISA. Fig 2.1 shows representative titration curves for several mAbs as described in the Materials and Methods. They detected a molecule in Western gel of brain extracts with Molecular Weight 33-48kDa, and of 43kDa in thymus extracts (Fig.2.2 Thymus & Brain). These mAbs also stained CHO transduced by a pBK vector encoding OX-2 (see below).

Data in Table 2.2 show FACS analysis for several of these mAbs using fresh cells (PBL or spleen dendritic cells, from normal or pv immunized mice). The data are summed over several independent analyses, using a number of mAbs directed to mouse OX-2. Representative FACS staining of spleen dendritic cells is shown in Fig.2.3.

It is clear from this Table (2.2) that PBL in all mice tested contained some 1.3%-2.5% OX-2+ cells by FACS analysis, and that spleen adherent cells similarly contained 4%-8% OX-2+ cells. As confirmation of our previous work, we also report that spleen adherent cells taken from C3H mice treated 4 days earlier by portal venous immunization with 20x10⁶ C57BL/6 bone marrow cells showed some 3.5-5 fold elevation in OX-2+ cells (see Table 2.2). Under these conditions we have reported specific increases in survival of subsequent allotransplanted cells/tissue (206).

2.4.2 Confirmation of the specificity of anti-OX-2 mAbs using CHO cells expressing OX-2:

A more definitive approach to confirm the specificity of the mAbs used for OX-2 used CHO cells transfected to express murine OX-2 on their surface. As described earlier, in the Materials and Methods. OX-2 was cloned and expressed on the surface of CHO cells using the strategy described in Fig.2.4 (a-c). Fig 2.5 shows immunohistology staining with Sigma Fast DAB of CHO cells transduced with control or OX-2 expressing pBK vector, using the (anti-OX-2) hybridoma 3.6 for staining.
2.4.3 Ability of anti-OX-2 mAbs to modulate cytokine production in MLR in vitro:

To address the functional importance of OX-2 expression on murine immune responses we asked whether these mAbs can modify the immune response (as assayed by cytokine production) of cells stimulated in an allogeneic mixed leukocyte reaction (MLR) in vitro. We have previously shown that cells taken from mice pretreated by portal allogeneic immunization produce predominantly type-2 cytokines, and that an anti-rat OX-2 mAb could apparently reverse this polarization in cytokine production (and indeed abolish the increased graft survival seen in such mice)(195). Data in Table 2.3 confirm these results using 3 independent anti-OX-2 mAbs previously screened by ELISA/Western Blots.

2.4.4 Reversal of polarization in cytokine production, and graft survival, after in vivo infusion of anti-OX-2 in pv immunized mice:

Previous studies have shown that in pv immunized mice, skin allograft survival is prolonged, in association with a polarization to type 2 cytokine production (see above, Table 2.3). In a preliminary report, using a cross-reactive anti-rat antibody, we showed that anti-OX-2 given from the time of transplantation, could reverse these changes (195). In order to assess the effect of infusion of a rat anti-mouse-OX-2, given at different times post transplantation, on cytokine production/graft survival, we performed the following study.

Groups of 6 C3H mice received pv immunization with C57BL/6 bone marrow derived dendritic cells (see Methods). Control groups received iv immunization or saline only (pv). 3 days later all mice received C57BL/6 skin grafts. Thereafter various groups of pv immunized mice received either no further treatment, infusion of control rat Ig, or 5 iv infusions of 100μg/mouse anti-OX-2 (clone 3.6) at the following times: days 0,2,4,6 and 8 (group pv0 in Figure 2.6); 3,5,7,9 and 11 (group pv3 in Figure 2.6); or 7,8,9,10 and 11 (group pv7 in Figure 2.6). Skin graft survival was followed daily, with data shown in Figure 2.6. In a separate study, we analyzed the same groups using 3mice/group, but sacrificed all mice at 14 days post transplantation. Spleen cells were harvested and restimulated in vitro with irradiated C57BL/6 spleen cells. Cytokine assays were performed on supernatants at 60hrs (see data in Table 2.3).
There are two key features to note in the data of Figure 2.6 and Table 2.4. Firstly, the increased graft survival and polarization to type-2 cytokine production seen following pv immunization is indeed reversed by anti-OX-2 treatment. Most interesting, this reversal is seen even when anti-OX-2 is infused very late (beginning 7 days following grafting and 10 days following pv immunization). This implies that the role of OX-2 in altering these functions is dependent upon continued functional expression (at least beyond day 8-post transplantation).
Fig. 2.1 Representative titration curves for several anti-OX-2 mAbs (note OD in control wells with second antibody only was <0.020 in all cases)
Fig. 2.2 Western Blot with rat/mouse anti-OX2:

a) Western Blot using commercial anti-rat OX-2 Ab and goat anti mouse IgG Ab; a rat thymus extract shows a positive band at Mol.Wt. = 42-47 KDa (positive control).

b) Western Blot using rat anti mouse OX-2 (Clone 3.6) mAb and goat anti-rat IgG HRP Ab; a mouse brain extract shows a band at Mol.Wt. ≈ 33 KDa.

c) Western Blot using rat anti-mouse OX-2 (Clone 3.6) mAb and goat anti-rat IgG HRP Ab; a mouse thymus extract shows a band at Mol.Wt. ≈ 48 KDa.

In all cases, staining with second antibody alone gave no detectable signal (data not shown).
Fig 2.3 Representative FACS staining of spleen dendritic cells with 3.6 (anti-OX-2) or control Ig, as well as FITC anti-DEC205, FITC B-7.1 or FITC B7.2.

Fig. 2.3 FACS stain of spleen DC from normal (control) or pv immunized mice. Mabs used were as shown. Data indicate % FITC positive cells.
Fig 2.4 A cloning strategy to produce a vector encoding full-length murine OX-2

a) PCR product of the open reading frame (ORF) of the MRC OX-2 that has a BamH1 restriction site at the 5’ end and a XbaI site at the 3’ end.

b) The circular plasmid, pBK-CMV vector, of 4.5Kb and containing the mouse OX-2 insert, of 858bps. Total size is 5.376Kb after ligation and miniprep plasmid purification.

c) Transformed DH10β-E. coli with the murine OX-2 that have been selected with kanamycin and X-gal. Extracted DNA has two bands representing 4.5Kb(vector) and 858bp (OX-2) after digestion with BamH1 and XbaI restriction enzymes.
Fig 2.5 An immunohistochemical staining with Sigma Fast DAB for the CHO that express OX-2 on their surface after stable transfection with pBK vector.

(a) Negative control
CHO, transduced with only pBK vector.

(b) CHO cells transfected with pBK vector contained the OX-2 construct.
Fig 2.6 Reversal of increased skin graft survival by anti-OX-2 (3.6)

All groups of 6 C3H mice received PV immunization with C57BL/6 bone marrow derived dendritic cells, except the control group shown (iv immunization). 3 days later all mice received C57BL/6 skin grafts. Thereafter mice received iv infusions of control rat Ig or anti-OX-2 (100µg/mouse x 5 doses). The control Ig and pv-group 0 received injections on days 0, 2, 4, 6 and 8 post transplant. Pv-group 3, and pv-group 7 received injections of anti-OX-2 on days 3, 5, 7, 9 and 11, or 7, 8, 9, 10 and 11 respectively. Data show skin graft survival for each group. Groups O and ● show different survival from all other groups (Mann-Whitney U-test, p<0.05).
**TABLE 2.2**

FACS staining of PBL and spleen adherent cells in mouse, using anti-OX-2 mAbs

<table>
<thead>
<tr>
<th>Donor (^b)</th>
<th>mAb</th>
<th>Percent stained cells (^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>PBL</td>
<td>Spleen</td>
</tr>
<tr>
<td>NONE</td>
<td>3.5</td>
<td>1.9±0.4 6.7±2.1</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>1.7±0.4 5.2±1.6</td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>1.4±0.4 4.2±1.4</td>
</tr>
<tr>
<td>PV immune</td>
<td>3.5</td>
<td>5.9±1.5 20±4.1</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>5.2±1.4 17±3.6</td>
</tr>
<tr>
<td></td>
<td>2.8</td>
<td>4.7±1.4 15±3.3</td>
</tr>
</tbody>
</table>

**Footnotes:**

a. Fresh cells were obtained from adult (8-10 week) mouse donors. The same 3 separate tissue donors were used for each mAb tested.

b. Donor pretreatment refers to infusion of allogeneic bone marrow cells into the portal vein (C57BL/6 for C3H mouse donors) 4 days before harvest of PBL or spleen (see text and (206)).

c. Arithmetic mean (±SD) for percent cells stained in 3 independent assays. Control antibodies (FITC anti-rat IgG for anti-mouse mAbs) gave no significant staining above background (<0.2%).
**TABLE 2.3**

Type-1 cytokine production in MLR cultures is increased by anti-OX-2 mAbs

<table>
<thead>
<tr>
<th>mAbs in culture</th>
<th>Cytokine levels in culture supernatants&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ELISA assays (murine only)</th>
<th>Bioassay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>IL-2</td>
<td>INFγ</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>350±55</td>
<td>35±18</td>
</tr>
<tr>
<td>3.6</td>
<td></td>
<td>915±155*</td>
<td>117±25*</td>
</tr>
<tr>
<td>2.8</td>
<td></td>
<td>855±155*</td>
<td>105±28*</td>
</tr>
<tr>
<td>control Ig</td>
<td></td>
<td>370±75</td>
<td>36±11</td>
</tr>
<tr>
<td>No pv immunization</td>
<td></td>
<td>710±145</td>
<td>108±23</td>
</tr>
</tbody>
</table>

**Footnotes:**

a. MLR cultures were set up as described in the Materials and Methods in triplicate for each mAb. Responder spleen cells were from mice treated 4 days earlier by portal vein infusion of C57BL/6 bone marrow cells, except for the last row where responder cells were from non-injected C3H mice. mAb was added as a 30% supernatant concentration. Supernatants were harvested for cytokine assays at 60 hr.

b. Data show arithmetic means (±SD) for each mAb. All supernatants were assayed for a number of cytokines by ELISA, and for IL-2/IL-6 using bioassays (proliferation of CTLL-2, B9 respectively). Note that cells incubated with isotype control Igs (non-reactive by ELISA or FACS) gave cytokine data indistinguishable from cultures incubated in the absence of mAb. P<0.05, compared with cultures without mAbs.
Table 2.4

Anti-OX-2 monoclonal antibody (3.6) reverses polarization in cytokine production in vivo in pv immunized mice.

<table>
<thead>
<tr>
<th>mAbs in vivo*</th>
<th>Cytokine levels in culture supernatants b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IL-2</td>
</tr>
<tr>
<td>Saline pv</td>
<td>980±155</td>
</tr>
<tr>
<td>iv immunized</td>
<td>1050±18</td>
</tr>
<tr>
<td>pv immunized</td>
<td>220±45</td>
</tr>
<tr>
<td>None (Control Ig)</td>
<td>250±75</td>
</tr>
<tr>
<td>3.6- group pv0</td>
<td>525±115*</td>
</tr>
<tr>
<td>3.6- group pv3</td>
<td>755±155*</td>
</tr>
<tr>
<td>3.6- group pv7</td>
<td>970±175*</td>
</tr>
</tbody>
</table>

Footnotes:

a. MLR cultures were set up in triplicate as described in the Materials and Methods. Mouse responder spleen cells were harvested at 14 days post C57BL/6 skin grafting from mice treated 3 days before grafting by pv or iv infusion of C57BL/6 bone marrow derived dendritic cells (see Methods), along with monoclonal anti-OX-2, 3.6 (see Legend to Figure 2.6 for sequence of infusion of antibody in groups pv0, pv3 and pv7). Supernatants were harvested for cytokine assays at 60 hr.

b. Data show arithmetic means (±SD). Assays for IL-2, IL-4 and IFNγ cytokines were by bioassay (measuring proliferation of CTLL-2 (IL-2), CT4.S (IL-4) and inhibition of proliferation of WEHI 279 (IFNγ) respectively), while for IL-10 an ELISA assay was used (see Table 2.2).

* p<0.05, compared with mice receiving control Ig.


2.5 Discussion:

Unresponsiveness to donor renal allografts following antigen-specific portal vein pre- or peri-transplant immunization is associated with the preferential activation of type-2 rather than type-1 cytokine producing cells (108, 214). T cell activation depends not just upon the delivery of signals via the TCR (generally in the form of antigen-MHC), but also on the appropriate delivery of costimulatory signals from antigen presenting cells (APC) (215), along with the cytokine milieu in which stimulation occurs. In the model we have used, IL-12 and/or IFNγ, and conversely IL-4, IL-10 and/or IL-13, are reported to regulate in reciprocal fashion the activation of type-1 and type-2 cytokine producing cells (214).

Using a PCR-based subtraction hybridization method in which amplification of "nontarget" DNAs in the PCR reaction is suppressed by attaching long inverted terminal repeats to the "tester" cDNA, we reported evidence which implicated a novel molecule, OX-2, in the regulation of graft rejection following portal vein immunization (195). In this preliminary report the evidence for involvement of mouse OX-2 in graft survival depended upon an unexplained cross-reactivity between mouse and rat OX-2 (using a mAb to rat OX-2) (195). In the data in this report we confirm that using species specific mAbs to mouse OX-2, that mAbs to the molecule detected on the surface of (host) dendritic cells may play a role in regulating cytokine production after allostimulation in vitro, and more particularly that functionally blocking OX-2 expression leads to enhanced IL-2 production (a type-1 cytokine) after allostimulation (Table 2.3). Borriello et al. also recently reported that OX-2 expression was not a costimulator for induction of IL-2 and IFNγ synthesis (203). Our data imply it is in fact a negative signal for type-1 cytokine production. In mice preimmunized by the pv, as reported earlier, there is a 4-fold increase in OX-2 expressing cells in PBL and spleen, and a reversal of polarization in cytokine production (from type-2 cytokines to type-1 cytokines) after stimulation of cells in the presence of OX-2 (see Tables 2.2, 2.3 and Fig 2.3) (195).

In a preliminary study we investigated whether continual expression of OX-2 was needed for prolongation of graft survival, and/or polarization of cytokine production. Our data (Figures 2.6 and Table
show that in fact both skin graft survival (induced by pv immunization) and polarization of cytokine production to type-2 cytokines, was reversed by infusion of anti-OX-2, as reported earlier (195). Somewhat surprisingly, however, we found the optimum time to produce this effect was to infuse anti-OX-2 at later times post grafting (beginning at 7 days post transplantation in the Figure/Table shown) rather than immediately following grafting. This implies that continued expression of OX-2 is needed for the increased survival seen, and may reflect ongoing delivery of negative signals, and/or multiple effects of OX-2 expression at different stages of development of the allo-immune response.

Taken together we believe our data support the previous studies suggesting that increased expression of OX-2 following portal vein immunization is explained by an important negative signaling role for this molecule. We anticipate that modulation of OX-2 expression may have clinical relevance in a number of human diseases, including infectious disease, autoimmunity, transplantation and malignancy.
CHAPTER 3
3.1 General discussion

Rejection is the primary cause of graft loss following transplantation. This is countered clinically by the use of potent non-specific immunosuppressive drug therapy. However, infection, malignancy and non-specific drug toxicity have represented significant problems for this mode of therapy, and focused attention on the development of more specific regimes to promote graft survival. One technique, which has received attention in this regard, has been the use of donor specific transfusion/immunization (DST). More recent studies, most particularly in experimental settings, have suggested that the route of immunization is of importance, with antigen delivered by the pv route being particularly tolerogenic.

Successful clinical organ transplantation has been described using pre-transplant transfusion (188, 216, 217). Under experimental conditions pv administration of soluble antigen was reported to induce specific tolerance in the late 60’s (218). More recently, foreign cells infused via the same route have been reported to induce donor specific tolerance across both major (164, 190, 195, 219) and minor (107, 108, 110, 220) histocompatibility barriers.

Extensive studies from our laboratory have shown that graft survival is associated with the activation of Th2 cells rather than Th1 cells, as judged from skin graft studies using multiple minor incompatible strains of mice (B10.BR or BALB.K into C3H/HEJ; C3H.SW into C57BL/6). This was assayed by quantitating production of type-1 cytokines (IL-2, IL-12, IFN-γ) vs type-2 cytokines (IL-4, IL-10, IL-13)(110). Similar results were seen using vascularized grafts transplanted across major histocompatibility barriers (195, 206). Despite these relatively clear-cut results, it should be noted that the notion that a Th1 to Th2 switch is universally responsible for delayed graft rejection is not without controversy. It has been shown that in IL-2 knockout mice, the deficiency in IL-2 production does not protect from islet graft rejection (116). Interestingly, IFN-γ is expressed strongly in the grafts of IL-2-deficient mice alongside IL-4 and IL-10, suggesting that in these animals no generalized shift towards a Th2 dominated response has occurred. Furthermore, again using knockout mice, it was found that
tolerance could be readily established using experimental protocols despite the absence of IL-4 production (221). More recently, in a model of tolerance to rat cardiac grafts which results from use of non-depleting anti-CD4 antibodies, Hall et al found no correlation between cytokine production patterns and long-term graft acceptance (222). These data suggest that the exact role of Th1 vs Th2 in transplantation is much more complex than at first thought, and our level of understanding still awaits further refinement.

We postulated, based on analyses such as those just described, that there were other differentially expressed genes which also played a critical role in graft acceptance seen after pv immunization. Using a novel and powerful technique, involving PCR based subtractive hybridization for identifying and isolation of differentially expressed genes (195), a number of novel transcripts were detected. One of these was found to encode the murine homologue of a gene described in 1982 by Barclay et al, OX-2 (202).

OX-2 is a molecule belonging to the Ig supergene family, and it has significant homology with members of the murine B7 family (203). This was itself of interest because the B7 class of molecules have been reported to afford the primary molecular interactions providing “costimulatory” signals for effective T cell activation (66, 67). We reported that OX-2 expression was increased within 2-3 days of pv immunization, as described by FACS analysis and Western Blots using material isolated from the liver/spleen of grafted animals (195). Since OX-2+ cells infused via the pv route still induced tolerance, it was suggested that the "OX-2 signal" detected in the spleen of the pv immunized mice derived from new expression (in host cells) rather than from infused OX-2+ cells.

A major criticism raised to the preliminary data we reported was that we had relied on an unexpected cross-reactivity between murine and rat OX-2, as defined by antibodies raised to rat OX-2, to define an immunosuppressive role for OX-2 expression. One specific aim of the current project was to raise and characterize mAbs specific to murine OX-2.

In a recent study in which the rat OX-2 gene was used to transfec CHO cells for costimulation of mouse cells Borriello et al showed that in contrast to B 7.1 mediated costimulation, OX-2 expression did
not result in detectable levels of IL-2, IL-4 or IFN-γ (203). Moreover, OX-2 transfectants did not bind the soluble receptor reagents of the B7 costimulatory pathway, namely CD28-Ig and CTLA4-Ig. However, OX-2 CHO cell transfectants were capable of providing a potent second signal to T-cells that receive signaling through the TCR by either MHC-peptide complex or anti-TCR mAb, and induced these cells to proliferate (203). Additional data from the same group showed that OX-2 costimulation was not inhibited by CTLA4-Ig, unlike B7.1 mediated costimulation, but was readily inhibited with an anti-OX-2 monoclonal antibody. Their conclusion from these data was that OX-2 is a T-cell costimulatory molecule which acts through a “non”-B7: CD28/CTLA4 pathway, and which leads to functionally distinct consequences (compared with B7 stimulation), as reflected by the resulting cytokine profile (203).

We have hypothesized that far from being a different costimulator molecule for T cell activation, OX-2 is a functionally important molecule in tolerance induction/immunoregulation, and plays a key role in the increased graft survival seen in our transplant model. We suggested that the tolerizing (negative) signal delivered to the T-cells can be monitored, in at least some instances, by the production of type 2 cytokines (195). Accordingly a second goal of the current work was to characterize the function and importance of expression of OX-2 molecule in graft survival using the newly developed anti-mouse OX-2 monoclonal antibody. In particular we wanted to confirm that anti-OX-2 did indeed block increased survival following pv immunization, and to examine the kinetics of expression of OX-2 (for operational tolerance induction) as defined by examining whether anti-OX-2 reversed increased graft survival only if given early following pv immunization, or whether continued expression was needed for prolonged survival.

3.2 Specific novel results described in this thesis:

In chapter 2, I have presented the results of ELISA screening for different mAbs raised to murine OX-2 in outbred rats –see Fig 2.1. Confirmation that these antibodies did indeed detect murine OX-2 was obtained using three different approaches.
In the first strategy, Western blots were performed using both mouse brain and thymus extracts with the relevant mAbs and the appropriate control antibodies (Fig 2.2 a, b, and c). These results revealed a difference in the size of the band detected in the two tissues, with mouse brain extracts having a band at molecular weight \( \approx 33 \) KDa, while the band in mouse thymus extracts migrated at \( \approx 48 \) KDa, as determined by polyacrylamide gel electrophoresis in sodium dodecyl sulfate. These results are in good agreement with the previously published data of Barclay et al for rat OX-2 (202). Differences in the apparent size of this molecule in different tissues is probably a function of differential glycosylation (195, 202). Thus while the amino acid compositions of brain and thymus OX-2 antigens were identical, carbohydrate compositions showed that OX-2 was highly glycosylated in both tissues, with brain OX-2 antigen containing 24% and thymocyte OX-2 antigen 33% by weight of carbohydrate. In addition, thymocyte OX-2 contained higher levels of galactose and sialic acid but less fructose than brain OX-2 (202).

As a second confirmatory approach, FACS analysis was performed using putative anti-OX-2 mAbs and fresh peripheral blood leukocytes (PBL) isolated using mouse lymphopaque (Cedarlane laboratories), or fresh spleen dendritic cells isolated after adherence and overnight incubation, following techniques described in detail elsewhere (195). Both cell preparations stained with anti-OX-2 mAbs at numerical values consistent with independent analyses of their dendritic cell content (Table 2.2 and Fig 2.3).

A final, more definitive, strategy for confirmation that these mAbs did indeed detect murine OX-2 used a vector construct I prepared in which full length OX-2 was inserted into a stable (pBK) expression vector, and CHO cells were transfected with this vector. The data obtained showed stable transfection of OX-2 on the surface of CHO cells as defined by one of these mAbs (3.6), with no detected expression in cells transfected with control vector (Fig 2.5).

Previous data from our laboratory have shown that pv infusion of DCs is a potent means of inducing tolerance in experimental animals (206). The most potent cell implicated in this process has been found to be one expressing (or induced to express) OX-2. In the next series of experiments I asked
whether the newly produced mAbs to murine OX-2 could modify the immune response of allostimulated responder cells in vitro. The response was monitored by measuring cytokine production in spleen cells stimulated in an allogeneic mixed leukocyte reaction (MLR). The mouse responder spleen cells (C3H) that were used were derived from mice treated 4 days earlier by pv infusion of C57BL/6 cells, and thus showed a preferential polarization to type-2 cytokine production rather than type-1 cytokine production.

While there was no change in cytokine production seen when these cells were incubated with isotype control Igs, incubation in the presence of the anti-OX-2 mAbs reversed the polarization in cytokine production, with production of type-1 cytokines now being predominant (Table 2.3). These results are in accord with previous data from our laboratory using a mouse anti-rat OX-2 (commercial) mAb (195). In these studies it was shown that cells taken from mice pretreated by pv immunization produced predominantly type-2 cytokines, and that an anti-OX-2 mAb infused in vivo could reverse this polarization in cytokine production (and indeed abolish the increased graft survival seen in such mice) (195). Note that none of the data obtained to date indicate unequivocally a direct signaling role for OX-2 in the increased graft survival following pv immunization.

In the final study described in Chapter 2, I examined the necessity for continued OX-2 expression for prolonged graft survival after pv immunization. The hypothesis tested was that if OX-2 expression delivered an initial potent tolerizing signal, which re-directed immunity in pv immunized/grafted animals, then late infusion of anti-OX-2 (several days after grafting) might not reverse prolongation of survival. In contrast, if continual expression of OX-2 was needed for prolonged survival, I predicted that grafts would be rejected at a similar tempo (to controls) as soon as infusion of anti-OX-2 mAb began, with corresponding changes in cytokine production. The data for this study, along with an analysis of cytokine production in these mice, is shown in Figure 2.6 and Table 2.4 in Chapter 2. It is evident from these data that in fact optimal inhibition of modulation of graft rejection and of the polarization in cytokine production (in pv immunized, transplanted, mice) was seen when anti-OX-2 was infused at late times following pv immunization and transplantation, rather than immediately after transplantation. These data
imply that continued OX-2 expression is needed for prevention of rejection, and for polarization in cytokine production (to type-2 cytokines).

3.3 Summary of our current understanding of the mechanism of unresponsiveness following pv immunization

The mechanism(s) responsible for tolerance induction seen after pv immunization is still unclear. Some reports in the literature have suggested that neither clonal deletion nor active suppression is involved in pv induced tolerance (223). T cells become anergic when activated in the absence of costimulatory signals (83, 85). Accordingly, one could ask whether conditions leading to clonal anergy exist following pv immunization. PV immunization has been reported to induce hyporesponsiveness of CD8+ cells more readily than CD4+ T cells, which may be due to expression of MHC Class I, the ligand for CD8, but not MHC Class II or B7 molecules on donor cells trapped in the host liver. Clonal anergy can be corrected by the addition of exogenous IL-2 (83), though only one study to our knowledge has addressed this issue in pv immunized mice (195). We have provided evidence that there is a major role for OX-2 expression in the tolerance induction which follows pv immunization, though the mechanism(s) by which OX-2 expression produces these effects remain obscure.

It should be noted that both in the studies described in this thesis, and elsewhere, pv immunization alone does not lead to indefinite survival of allografts, whether vascularized (renal or small intestinal allografts) or non-vascularized (skin) grafts are studied. There are a number of possible explanations for this finding, not the least of which is that there is likely no single mechanism (deletion/anergy/suppression) for graft rejection per se, and interfering with any one may not suffice to produce indefinite survival. We also have discussed elsewhere (see Gorczynski, 1999-in preparation), that although OX-2 expression is associated with direct delivery of a negative signal to activated cells, even this may only produce optimum inhibition of rejection when given in the context of inhibition of other positive signals (e.g. by blocking B7-1/B7-2 or CD28, CTLA4 signaling, for instance).
Our laboratory reported several years ago that unresponsiveness in pv immunized animals could be adoptively transferred to naïve recipients by hepatic APC or, more interestingly, by γδ T cells (126, 224). Adoptive transfer of unresponsiveness implies that suppressor mechanisms, rather than anergy or deletion, are involved in tolerance. Note that both Waldmann et al and Hall et al have identified suppressor mechanisms in tolerant animals following infusion of non-depleting anti-CD4 mAbs (221, 222). A tolerogenic APC induced following pv immunization may be one preferentially expressing OX-2, as discussed above. The role of γδ TCR+ cells is less clear. However, a number of observations implicate γδ TCR+ cells in a variety of conditions associated with chronic stimulation/inflammation in vivo, including multiple sclerosis and arthritis (129-131). It is known that γδ TCR+ cells can be activated to produce a number of cytokines, including those that alter the Ag-presenting function of LCs, epidermal APC for (αβ) T cells (133).

Thus while the potential implications of the role of OX-2 expression in the preferential induction of γδ TCR+ cells following pv immunization remains to be explored. there are some points worthy of attention. Thus, note that data in Chapter 2 implies that continued OX-2 expression is needed for prolongation of graft survival, and/or polarization in cytokine production. This may reflect a role for OX-2 signaling at multiple stages in development of the allo-immune response (and its regulation) after pv immunization. It is interesting to speculate that the later stages of this process (e.g. those inhibited by anti-OX-2 delivered at days 3-7 post grafting-see Table 2.3) may indeed be manifest primarily at the level of γδTCR+ cell expansion/function.

To sum, the experiments described in this thesis have further highlighted the important role played by the OX-2 molecule in tolerance induction following pv immunization. The anti-murine OX-2 mAbs described will be an asset in future studies to explore the mechanism of, and role of OX-2 expression in, tolerance induced in pv immunized mice. Understanding the mechanisms by which unresponsiveness is maintained is essential for future clinical application of the approaches described.
3.4 Future studies suggested by my work:

1. Analysis of the tissue and cellular distribution of OX-2 following pv immunization.

Using a commercial mAb to rat OX-2 it was reported that OX-2 expression was increased on NLDC145+ (dendritic) cells in the liver/spleen within 2-4 days of pv immunization. Reagents to murine OX-2 will be used to confirm these findings. In addition, a more exhaustive analysis of the localization of OX-2 in tissues from grafted mice will be performed, using immunofluorescent techniques. Tissues will be co-stained with a variety of other (commercial) mAbs, including those to mouse dendritic cells (NLDC145), tissue macrophages (F4/80) and distinct subsets of dendritic cells (33D1). In a separate study our laboratory recently reported that OX-2 expression was seen on a subpopulation of NLDC145+ cells in vivo/in vitro. The tissue (sub) localization of these cells is unknown.

2. Is there a role for increased OX-2 expression in increased graft survival in other models, such as following xenogeneic grafts, and in protection from GvHD after bone marrow transplantation?

Our laboratory has reported that pv immunization affords prolongation of skin, renal and small bowel allografts, as well as skin and renal xenografts (107, 111, 213, 225). Furthermore, decreased acute GvHD and prolonged survival of animals receiving bone marrow transplants has been observed following pre-transplant antigen-specific pv immunization (213). The functional role of increased expression of OX-2 in these model systems will be explored as follows.

Using PCR specific primers for rat/mouse OX-2, we will examine evidence for increased expression of this antigen in these different models. In addition, the mAbs described above will be used to ask whether the inhibition of increased survival seen in pv immunized recipients translates into similar effects in these other systems. If, as our laboratory has predicted, OX-2 is a molecule with a general
immunomodulatory function in vivo, we predict its involvement in the prolongation of graft survival in all of our test models.

On a more general level, we propose that anti-OX-2 mAb will reverse tolerance even to nominal antigen, following pv (or oral) immunization. This could be tested in a model where the decreased anti-OVA antibody response, in mice fed OVA and then immunized with OVA, is reversed after infusion of anti-OX-2.

3. Confirmation of a definitive role for OX-2+ cells in tolerance induction:

OX-2 expression is increased after pv immunization, and anti-OX-2 mAb infusion reverses the increased graft survival following pv immunization. Confirmation that this expression of OX-2 is functionally important in the induction of increased graft survival in this model would come from studies using adoptive transfer of OX-2+ cells to induce graft tolerance. Preliminary data using this approach has been reported, using cells enriched by sedimentation analysis (195). However, a superior approach would be to use mAb-enriched cells isolated from pv immunized animals.

Anti-OX-2 mAb purified cells from pv immunized C3H mice (given C57BL/6 cells) would be adoptively transferred to naive C3H mice, which then subsequently receive C57BL/6 (specific) or DBA/2 (third party) skin grafts. OX-2+ cells can be enriched using panning procedures (estimated $5 \times 10^6$ cells/spleen of PV immunized mice based on FACS data to date). An alternative purification procedure uses FACS-sorting. Using this technology we would also be in position to answer the question whether OX-2 expressing cells inhibit an ongoing rejection response (using mice pre-grafted before transfer). Once again confirmation for an important role for OX-2 expressing cells in these phenomena would rely on administration of anti-OX-2 mAb to reverse the effects seen.

4. Examination of a role for OX-2+ cells in triggering $\gamma\delta TCR^+$ cells after PV immunization:

As noted above, our laboratory reported several years ago that unresponsiveness in pv immunized animals was associated with a preferential expansion of $\gamma\delta$ TCR+ cells (126, 224), which could adoptively transfer tolerance to naïve recipients. We have speculated that tolerogenic APC induced following pv
immunization may preferentially trigger γδTCR+ cells at the expense of αβTCR+ cells. Such tolerogenic APC may be ones expressing OX-2. In order to explore this possibility γδTCR+ cells, and αβTCR+ cells, isolated using the appropriate mAbs from pv immunized mice, will be restimulated in vitro with APC (dendritic cells) isolated from naïve vs pv immunized mice. This restimulation will occur in the presence or absence of differing amounts of mAbs to molecules implicated in the delivery of accessory signals to T cells, namely B7-1, B7-2, CD40 and OX-2. Our prediction is that there will be a differential sensitivity to these reagents in the different populations of T cells. Further confirmation of such results could come from studies using CHO cells transfected with these same molecules (B7-1, B7-2, CD40, and OX-2) as costimulators/corepressors of cytokine production in T cells activated by TCR cross-linking.
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